ULTRAVIOLET RADIATION DAMAGE IN HIGHER PLANTS

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Abstract

The effect of ultraviolet (UV) radiation on higher plants was investigated using physiological methods and electron microscopy. Damage by UV-B (280-320 nm) radiation to the plasmalemma of isolated protoplasts of Beta vulgaris L. and Hordeum vulgare L. was estimated using fluorescein diacetate. The protoplasts showed increasing damage with irradiation time. With delayed light emission as a probe for detecting damage to the photosynthetic membranes, it was found that UV-B radiation resulted in a decrease in the intensity of light emission rather than a change in the decay rate. At the ultrastructural level in Beta vulgaris leaves, the chloroplast membranes appeared most affected. In addition to disorganization of thylakoid membranes and apparent areas of disruption of the chloroplast envelope, a quasi-crystalline formation of the stroma was observed in UV-B-treated material. In contrast, in leaf sections of UV-C (254 nm)-treated leaves, membrane-bound crystalloids were detected in the cytoplasm. Action spectra for effects of UV radiation on the photosynthetic system were determined using variable fluorescence. Isolated thylakoids (Spinacia oleracea L.) and intact leaves (Oxalis deppei Lodd and Elodea densa Casp.) were used. It appeared that UV absorbing compounds in the epidermis are important in protecting the photosynthetic system, but that sensitivity differences exist also at the thylakoid level. In UV- and non-treated material the fluorescence transients could be resolved into two (Elodea, Oxalis) or three (Spinacia) components. The amplitudes and rate constants of these components were differently affected by UV radiation among the different species. A comparison between the effects of blue light and UV radiation on fluorescence induction kinetics of photosystem II in Oxalis suggested that different mechanisms were involved. From analyses done on poisoned (DCMU-treated) and non-poisoned leaves it seems that the reaction center, as well as components on both the oxidizing and reducing sides of photosystem II are affected by UV radiation.

Key words

Delayed light emission, damage, fluorescence induction, photosynthesis, photosystem II, ultrastrucure, ultraviolet radiation

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DOCTORAL DISSERTATION

BY

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ULTRAVIOLET RADIATION DAMAGE IN HIGHER PLANTS

Janet F. Bornman

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ULTRAVIOLET RADIATION DAMAGE IN HIGHER PLANTS

INTRODUCTION

1. The ozone layer

With the formation of an ozone layer in the stratosphere (10-50 km above ground level) life on earth was shielded from the lethal short-wavelength solar radiation, since this radiation (UV-C, <280 nm, and much of the UV-B between 280 and 310 nm) is effectively absorbed by ozone. Scattering and absorption of aerosols also attenuate solar UV-B and UV-C, although to a much lesser extent. Ozone itself is generated when shortwave ultraviolet radiation (< 242 nm) splits an oxygen molecule and the liberated oxygen atoms combine with another oxygen molecule to form ozone. A dynamic equilibrium exists naturally with an approximate balance between ozone formation and destruction. The destruction of ozone is effected by chlorine oxides, nitrogen and hydroxy radicals and atomic oxygen.

In the 1970's concern arose that anthropogenic pollution might interfere with the ozone chemistry as a result of an increased release of certain catalytic compounds into the atmosphere. These compounds derive mainly from chlorofluorocarbons (e.g. freons, used

extensively as refrigerants and as propellants for aerosol sprays), nitrogen fertilizers, high-flying aircraft and ground vehicles. In order to estimate the possible ozone reduction and concomitant increase in UV radiation to ground level, the combined action of both destructive and constructive processes must be considered. It has been estimated that the depleting effects of chlorofluorocarbons and nitrogen oxides coupled with the ozone-producing effects of CO_2 (CO_2 leads to an ozone increase due to cooling of the stratosphere) and methane (which prevents the chlorine species from depleting ozone by converting these species to hydrochloric acid molecules, which are precipitated as acid rain) together will result in ca 3 % decrease in ozone by the year 2080 (Brasseurl and De Rudder 1985).

The importance of the ozone layer (corresponding to a layer ca 3.0 mm thick at STP) lies both in its capacity as an effective UV filter and also as a stabilizer of climate. An increase in UV radiation at ground level as a result of a reduced ozone layer may lead to increases in malignant melanomas in humans as well as adversely affecting plant life. Research has therefore centered around the problem of how the ozone layer should be protected, estimation of the damaging effects of an increased UV level, and how the detection of these effects can be used as an early warning system.

2. UV radiation

Although UV radiation (<400 nm) constitutes only about 7 % of the solar energy reaching ground level with the UV-B (280-320 nm) component making up < 0.3 % (Diffey 1985) of the total solar radiation, biologically important macromolecules absorb effectively in the UV-B spectral range which can result in marked damage. Since the large ozone absorption coefficient at wavelengths less than 280 nm (UV-C) efficiently prevents radiation of these wavelengths from reaching ground level, and since the small coefficient at longer wavelengths is unlikely to influence the solar flux much, any reduction in the ozone layer would affect solar flux at the shorter UV-B wavelengths most (Caldwell 1981), in addition to an effect in the infrared region. However, a given decrease in ozone concentration is not proportional to the increase in biologically damaging radiation. A "radiation amplification factor" of two has been estimated, meaning that for every percent decrease in ozone concentration, a 2 % increase in biologically damaging radiation will result (NAS 1979). However, the radiation amplification factor is dependent on the action spectrum for damage, as well as on the daylight spectrum. Therefore different types of damage, as well as different solar elevations, etc, give different radiation amplification factors.

3. Sensitivity of plants to UV radiation

Recent studies have indicated that present levels of UV radiation have a detrimental effect on certain sensitive plants (Bogenrieder and Klein 1977, Sisson and Caldwell 1977, Teramura et al. 1980, Teramura and Caldwell 1981, Worrest 1983). Although information on the effects of UV radiation on plants has accumulated in recent years, the questions involving possible UV targets and the precise mechanisms of damage have remained unanswered. This is not surprising since many indirect effects of UV damage obscure the primary causes. Injury to the whole organism by UV radiation is a consequence of damage to its constituent cells and in turn to biologically important molecules. Several different chromophores are likely to be involved. The genetic material is a critical component of a cell, and even the shorter wavelengths of the UV-B spectral range with present ozone concentrations may induce damage (NAS 1979). Although the cellular repair mechanisms reduce the vulnerability of the cells, an increase in the UV-B component may interfere with these mechanisms (NAS 1979).

The marked variation of higher plants in their sensitivity to solar UV radiation is not fully understood, although it is known that molecular repair mechanisms (mostly studied at the DNA level) are involved. Acclimation of plants to UV radiation can also occur, and may involve increased production of flavonoids and related phenolic compounds in the epidermal and

mesophyll cells. These compounds may effectively attenuate UV fluence at sensitive physiological targets. However, since flavonoids are mostly concentrated in the cell vacuoles of the epidermis and since the epidermis is not a uniform filter, UV radiation must be able to penetrate to the deeper-lying tissues (Caldwell et al. 1983). It is therefore of economic and ecological importance that the harmful effects of solar UV-B radiation be carefully analyzed.

Several effects of UV-B radiation on plants have been reported with photosynthesis being one of the major processes affected. It is suspected that several types of stress may result from UV radiation and that these stresses interact with each other thus leading to a variety of symptoms whereby some responses may be enhanced or masked by others (Teramura 1985). Fox and Caldwell (1978) showed that the competitive balance of different species can change with UV stress. There is also evidence (Gold and Caldwell 1983) that even at ambient solar UV-B radiation a shift occurs in the competitive interaction of some species as compared to when the UV-B component is filtered out.

Specific effects at the molecular level of the cell include membrane alterations and inhibition of metabolic pathways. Damage to membranes can result in a chain of altered events which may involve malfunctioning of certain channels and electrogenic membrane pumps; membrane depolarization and changes in cytoplasmic pH may

also occur (Murphy 1983).

4. UV damage to the photosynthetic system

Photosynthesis is directly affected by UV radiation, and this may reduce crop productivity. Extensive work has also been done on the partial reactions of photosynthesis as well as on specific membrane components and enzymatic processes in an attempt to identify the main target(s) of UV radiation. To date there is still speculation concerning the mechanism of UV inhibition of the photosynthetic system (e.g. Mantai and Bishop 1967, Noorudeen and Kulandaivelu 1982, Renger et al. 1985).
In order to obtain a better understanding of the effect of UV radiation on plants, the following investigations, numbered I-V on page 7, were carried out.

List of papers included in the thesis

I Bornman, J.F., Bornman, C.H. & Björn, L.O. 1982. Effects of ultraviolet radiation on viability of isolated Beta vulgaris and Hordeum vulgare protoplasts. - Z. Pflanzenphysiol. 105: 297-306.

II Bornman, J.F., Evert, R.F. & Mierzwa, R.J. 1983. The effect of UV-B and UV-C radiation on sugar beet leaves. - Protoplasma 117: 7-16.

III Bornman, J.F., Björn, L.O. & Akerlund, H.-E. 1984. Action spectrum for inhibition by ultraviolet radiation of photosystem II activity in spinach thylakoids. - Photobiophys. Photobiochem. 8: 305-313.

IV Björn, L.O., Bornman, J.F. & Olsson, E. 1985. Effects of ultraviolet radiation on fluorescence induction kinetics in isolated thylakoids and intact leaves. - In Stratospheric Ozone Reduction, Solar Ultraviolet Radiation and Plant Life (R.C. Worrest, ed.) Springer-Verlag. ISBN 13875-7. (In press).

V Bornman, J.F. & Björn, L.O. 1985. Inhibition of photosystem II by blue light and ultraviolet radiation: a comparison. (unpublished).

I Effects of ultraviolet radiation on viability of isolated *Beta vulgaris* and *Hordeum vulgare* protoplasts

Protoplasts were isolated from two economically important crop plants (*Beta vulgaris* and *Hordeum vulgare*) and two methods were used to examine the effect of UV radiation. Damage to the outer membrane of the cell, the plasmalemma, was estimated using the vital stain, fluorescein diacetate (FDA). The FDA molecule is taken up by intact protoplasts and hydrolyzed by endogenous esterases. Fluorescein is thereby released and its bright yellow-green fluorescence can be viewed by fluorescence microscopy. Degree of damage to the plasmalemma was semi-quantitatively assessed based on the fluorescent colour changes which occur with increasing membrane damage (Figs 4-7, p. 302 of paper I).

UV-B radiation affected the plasmalemma of the protoplasts of the two species to varying extents. Under the experimental conditions *Beta vulgaris* (a dicotyledon) was more sensitive to UV-B radiation than was *Hordeum vulgare* (a monocotyledon).

The effect of UV-B radiation on photosynthetic membranes was investigated in the same study using delayed light emission (DLE) as a probe. Strehler and Arnold (1951) first reported DLE from chlorophyll. DLE is composed of components characterized by different decay rates ranging from nanoseconds to minutes. This delayed chlorophyll fluorescence comes from radiative de-excitation as a

consequence of recombination of positive and negative charges. Although DLE has an emission spectrum similar to prompt fluorescence from chlorophyll a (indicating that DLE also originates from de-excitation of Chl a in the singlet excited state), the means by which the singlet excited state of Chl a is populated differs. DLE is thought to come from Chl a molecules close to the reaction center when charges recombine in the center. In contrast, prompt fluorescence arises from relaxation of the antenna Chl a molecules that have absorbed quanta (Govindjee and Jursinic 1975).

In most of the work done on DLE in higher plants photosystem II (PS II) is activated by light absorption prior to measurements. Light emission from PS II can also be experimentally induced, as was done in the present experiments, by using far-red light which preferentially excites PS I. The resultant slow decay from PS II is a consequence of electrons recombining with Chl a near the PS II reaction center (Björn 1971). The way in which this occurs is probably dependent upon a number of processes involving hydrogen ions and the coupled electron flow, and may be explained as follows:

Far-red excitation of PS I induces cyclic electron transport which in turn drives hydrogen ions to the inside of the thylakoid membrane. This accumulation of hydrogen ions, i.e. the proton motive force which results, causes hydrogen ions to move out of the thylakoids and this reverse flow causes concomitant electron flow from PS I towards Q (a primary electron

acceptor of PS II). These electrons then combine with the positive charges always present in the water oxidizing system after a dark incubation (because of spontaneous equilibration to 25 % S_0 + 75 % S_1 states) and the excited chlorophyll so created emits light.
Using this method for studying DLE, the changes caused by UV radiation on the photosynthetic electron transport chain were evaluated.

Although the method using vital staining for determining damage to the plasmalemma and that of DLE cannot be directly compared, it was of interest to assess UV damage to two different systems.

II The effect of UV-B and UV-C radiation on sugar beet leaves

Since the integrity of membrane systems is necessary for coordinated cell and tissue function, both physiological and ultrastructural studies are of importance in determining the effect of UV radiation on membranes.

Ultrastructural investigations carried out on Beta vulgaris leaves that had been exposed to UV radiation during growth revealed that the chloroplast was the most sensitive organelle. Of particular interest were the effects resulting from UV-B irradiation because of its ecological importance. UV-C was, however, also included as an indicator of severe damage, although one must be cautious when making comparisons between effects of radiation in the two wavelength ranges without more extensive data from experiments with various fluences. UV-C radiation resulted in marked structural damage with excessive starch accumulation in chloroplasts (Figs 3-4, p. 10 of paper II) and membrane-bound crystalloids in the cytoplasm of many cells.

In UV-B treated leaf tissue, the most striking effects were discerned in what appeared to be disruptions in the chloroplast double membrane (Fig. 10, p. 14 of paper II), and in some of the leaf sections studied, the quasi-crystalline appearance of the chloroplast stroma (Fig. 11, p. 15 of paper II).

From the electron microscopy studies, an assessment of the effect of UV radiation points to the membranous system of the cell as the obvious target, with the chloroplast membranes being particulary vulnerable. Possibly membrane proteins and lipids are the primary targets, but the effects may be indirect. In this respect it has been suggested that UV effects on membranes resemble those of advanced senescence (Skokut et al. 1977).

From the results of papers I and II it is not surprising that physiological processes are adversely affected by UV radiation given the structural alterations incurred.

III Action spectrum for inhibition by ultraviolet radiation of photosystem II activity in spinach thylakoids

Variable fluorescence from Chl a of PS II was used for determination of an action spectrum for UV-inhibition of PS II in thylakoids of Spinacia oleracea. The results obtained using the rise time of variable fluorescence, a parameter not employed before in determining an action spectrum for PS II inhibition by UV radiation, agreed fairly well with that of Jones and Kok (1966). These authors measured inhibition of photoreduction of dichlorophenol indophenol (reflecting the Hill reaction) in isolated chloroplasts.

Action spectra have been used to determine probable UV radiation targets. Reliable action spectra are also important for the computation of biologically active UV radiation in order to make more accurate assessments of the effect of ozone reduction.

Many of the action spectra reported so far differ in their slope of decline with increasing wavelength. It is therefore necessary to evaluate which of the mechanisms showing damage are of significance for higher plants exposed to UV radiation. Action spectra showing a slow decline with increasing wavelength (as in Jones and Kok, 1966, and also in paper III) probably do not reflect ecologically significant inhibitions, since if they did, this would reflect only a small latitudinal gradient of effective solar UV radiation.

In an American study crop plants from temperate and tropical latitudes were screened for sensitivity to UV radiation. Low latitude species were found generally to be able to withstand elevated UV radiation levels, whereas more than 50 % of those species tested from temperate regions were sensitive under the experimental conditions (A.H. Teramura and M.M. Caldwell, cited as unpublished in Caldwell et al. 1985). Two kinds of action spectra have been determined; those derived using monochromatic radiation and, only recently, those from polychromatic radiation. There is evidence that action spectra obtained for UV-induced photosynthetic damage using polychromatic radiation are steeper than those using monochromatic light sources (Caldwell et al. 1985). Extrapolation from experimental to natural conditions should therefore take into account the type of action spectrum being used. While polychromatic radiation may more closely approximate natural conditions, action spectra determined with monochromatic radiation can give valuable information on damage to specific chromophores without the complications of synergistic effects induced by different wavelengths given simultaneously.

In paper III the chlorophyll fluorescence induction curves, apart from being used for an action spectrum, yielded interesting results in themselves.
Fluorescence induction can be used as an intrinsic probe for changes in photosynthetic electron transport, since impairment of the photosynthetic system is reflected in the shape of the induction curves. This fluorescence

transient or Kautsky effect (Kautsky and Hirsch 1931) is the change in Chl *a* fluorescence yield with illumination time. At physiological temperatures most fluorescence is emitted from PS II, while PS I is only weakly fluorescent and does not give the Kautsky effect. The fluorescence yield depends (i.e. there is usually an inverse relationship) on the efficiency of photosynthesis and on the dissipation of light energy in non-radiative processes. However, it does not always follow that low fluorescence yield points to an efficient or undamaged photosynthetic system. Lesions or areas of altered molecular configuration may be induced, the consequences of which often result in a reduced fluorescence yield.

A distinction is made between constant (F_o) and variable (F_v) fluorescence, since not all of the Chl *a* fluorescence is an indicator of the electron transport through PS II reaction centers. Upon illumination after a period of darkness, the fluorescence yield immediately reaches an initial level (F_o, origin or O level). This is followed by a rise from F_o to a maximum level (P, F_{max} or peak; $P-O = F_v$).

Fast (0-5 s) and slow (several minutes duration) changes in the fluorescence induction have been distinguished. The fast changes, reflecting the photosynthetic electron transport rate, incorporate the so-called OIDPS (see Govindjee and Papageorgiou 1971) transient (in higher plants, algae, and isolated, complete chloroplasts). In

isolated thylakoids and chloroplasts lacking the outer envelope, the ID phase (initial; dip) may be indiscernable and P (peak) is not followed by S (steady state). A rise to M (maximum) followed by a decline to the terminal (T) steady state level occurs in intact plants and whole cells, and represents the SMT or slow change.

Under the experimental conditions of paper III, treatment by UV radiation lowered the rate of fluorescence rise and the maximum fluorescence (the amplitude of variable fluorescence was somewhat decreased). This suggested that electron transfer was being inhibited on the oxidizing or water-splitting side of PS II, or in the RC itself. Addition of hydroxylamine (an electron donor on the oxidizing side of PS II) only partly reversed this inhibition, and the control level of F_v was not fully restored. From the results obtained it appears that UV radiation affects the photosynthetic system at more than one location and in different ways.

IV Effects of ultraviolet radiation on fluorescence induction kinetics in isolated thylakoids and intact leaves

The influence of UV radiation on photosynthesis was studied further by following changes of the Chl *a* fluorescence induction kinetics in intact leaves of *Elodea densa* and *Oxalis deppei*, aquatic and terrestrial plants, respectively. Internal screening was also evaluated. In addition the previous action spectrum determined for spinach thylakoids (paper III) was compared to those for *Oxalis* and *Elodea* leaves.

The fluorescence induction curves of the plants investigated were resolved into two (*Oxalis* and *Elodea* leaves) or three (spinach thylakoids) components. These components were all exponential except for one of the components obtained from *Oxalis* leaves. In general the effect of the UV radiation used (280, 310, 320 nm) was to decrease the amplitude of certain of these induction components rather than drastically affect the rate (except in the case of *Elodea* leaves where the rate constant of the slow component was decreased more than three times as compared to non-irradiated material).

To date, numerous interpretations have been put forward in order to explain the fluorescence kinetics obtained upon illumination of a dark-adapted sample. Various models have been advocated to account for the

experimental results, although there is as yet no absolute agreement as to a single mechanism, and results and interpretations differ. It is probable, however, that different plant material and different experimental conditions may be some of the causes for these discrepancies. For Oxalis leaves the fluorescence equations (8) and (9) of Lavorel and Joliot (1972) were used to evaluate the fluorescence rise from O (origin) to P (peak), although it is not maintained that the model is necessarily correct (alternative models have been proposed by various investigators).

The way in which the photosynthetic pigments may be arranged in the thylakoid membranes has given rise to two basic model systems, both of which are discussed by Lavorel and Joliot (1972).

In the so-called unconnected or separate model, each photosynthetic unit (PSU) is associated with its own reaction center (RC) so that if a RC has received a quantum of energy (closed state), then a subsequent quantum arriving at the same RC will be lost as fluorescence. In this case exciton trapping is independent of the state of other PSUs, such that first order kinetics are expected where the O to P rise reflects the increasing number of units in the closed state.

The connected model on the other hand, includes the concept of interunit transfer of excitation energy. This is thought to come about in the following way. Groups of PSUs (called islets by Lavorel and Joliot), upon

receiving excitation energy will tend to grow at their edges as a consequence of the increased territory (due to the closing of traps) of the light-harvesting chlorophyll from adjacent islets so that there will be gradual merging of these islets (forming larger islands). Therefore, the probability, p, of exciton transfer among units will depend on the whole system of actively expanding islets and ultimately on the merging of the islets. Under these conditions the model predicts a slow fluorescence rise followed by a more rapid one. The experimental data are best modelled by a sum of an exponential (separate units) and a sigmoid (connected units) component.

The kinetics of the fluorescence induction in Oxalis leaves before and after treatment with UV were further studied (paper IV). The initial fast rise of the induction curve of Oxalis did not fit the kinetics of the connected model, but rather that of the separate model where independent PSUs would reflect an exponential fluorescence rise, with the primary acceptor of PS II being reduced in a one-quantum step. The initial rise was affected most by the UV radiation employed. The effect was seen in the decrease of the amplitude of this first component.

A criticism against the connected (quasi-statistical) model of Lavorel and Joliot is that it does not make allowance for energy transfer between PSUs with open

RCs (with oxidized primary acceptor), rather it considers these RCs as absolute traps. One of the alternative approaches involves a matrix or statistical model. In this model there is no limitation imposed among the connecting PSUs, so that in principle there is allowance for random walk of quanta. Using this matrix model as well as the idea of the separate or unconnected model (already mentioned), Melis and coworkers (Melis and Homann 1975, 1976, Melis and Duysens 1979) analyzed the area above the fluorescence induction curve. This area, according to Murata et al. (1966) reflects the degree of photochemical progress (the number of photons utilized for photochemistry from the onset of illumination). They found that in the presence of DCMU there was a fast, non-linear (termed α-component) and a slower first order phase (β-component). It was suggested that the induction rise was due to two photochemical events with different rate constants. This difference has been ascribed to the occurrence of two types of PS II centers, namely α and β centers, spatially separated in the grana and stroma regions, respectively, of the thylakoids. The β centers are seen as belonging to a large pigment matrix with interunit energy transfer, whereas the first order kinetics of the β-component suggest groups of separate PSUs.

The results obtained for Oxalis in paper IV, although analyzed differently, may also reflect the existence of two kinds of PS II.

V Inhibition of photosystem II by blue light and ultraviolet radiation: a comparison

Fluorescence transients from Oxalis deppei leaves were evaluated in terms of the effects of both blue light (440 nm) and UV radiation (280 and 310 nm). With the photon fluences employed it was found that UV radiation irreversibly inhibited the chlorophyll fluorescence rise when leaves were irradiated from the abaxial side. The leaves were insensitive to UV radiation from the adaxial side.

Blue light resulted in largely reversible photoinhibition of the fluorescence rise, with the adaxial leaf surface being slightly more sensitive to blue light than the abaxial side. It seems that the photosynthetic system reacts differently to exposure by blue light as opposed to UV radiation.

In general, the term photoinhibition has been used to describe a loss in photosynthetic efficiency due to exposure of plants to high visible radiation. This photoinhibition is known to occur even with ambient levels of solar radiation. Consequently plants must have evolved ways to cope with high intensity visible radiation. In this context, Arntzen et al. 1984 suggest that the rapid turnover in light of the 32 kdalton Q_B

apoprotein, which they implicate as the primary site of photoinhibition, is a result of its role in the photosynthetic electron transport chain. Although this may provide one way of efficient protection against high light intensities, damage to other sites along the electron transport chain is also likely.
Kyle et al. (1984) and Arntzen et al. (1984) found that only after further treatment with high intensity light was damage also detectable on the oxidizing side of PS II.

Other workers (e.g. Critchley 1981, Powles and Björkman 1982), using much less total photon fluence than Arntzen and coworkers, found the oxidizing side of PS II to be primarily affected. The fact that these authors used considerably lower doses of visible light, and obtained results indicative of primary damage to the oxidizing side of PS II, suggests that this effect may be overlooked when excessively high photon fluences are used. The effect of UV radiation has also been interpreted along lines similar to those of Arntzen and coworkers. However, it seems that from investigations so far, UV radiation influences the electron transport system at more than one location, and the oxidizing side of PS II, the RC itself, and the reducing side of PS II are likely targets which may be affected under different experimental conditions.

In paper V the fluorescence curves obtained after treatment with either blue light or UV-B radiation were

further evaluated using equations 8 and 9 of Lavorel and Joliot (1972). This was done in order to compare the effects of blue and UV radiation and was not intended as a mechanistic explanation of the induction curves.

Nevertheless, certain patterns emerged from the different light treatments. As presented in Tab. 1 (paper V), the main effect of blue light was to decrease the amplitude of the slow component, and in contrast to UV radiation, there was a decrease in the rate constant of the fast component. The rate constant of the fast component and the amplitude of the slow component (abaxial leaf surface) were most affected by the UV photon fluences used. These results differ somewhat from those presented in paper IV (Tab. 3). These latter results may not be as accurate, since the modeling was much more approximate (done largely by hand) and did not as closely simulate the experimental results (see Tab. 3 and Fig. 4, paper IV). In addition, the data are from one representative experiment (Tab. 3, Fig. 4, paper IV), while those from the present paper are mostly averaged from several experiments.

Results have shown that high intensity blue light and UV radiation both result in damage to PS II, but that different kinds of damage occur.

Abbreviations: Chl, chlorophyll; DCMU, 3,-(3,4-dichlorophenyl)-1,1-dimethylurea; DLE, delayed light emission; PS II, photosystem II; PSU, photosynthetic unit; Q, first stable electron acceptor of PS II; RC, reaction center; UV-B, ultraviolet radiation (280-320 nm); UV-C, ultraviolet radiation (< 280 nm).

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Effects of Ultraviolet Radiation on Viability of Isolated *Beta vulgaris* and *Hordeum vulgare* Protoplasts

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Summary

Estimates of viability as measured by vital staining with fluorescein diacetate were carried out on freshly isolated and partially aged (16-hour-old) *Beta vulgaris* and *Hordeum vulgare* mesophyll protoplasts following irradiation with UV-B. Damage to the photosynthetic system by UV-B was determined by delayed light emission (DLE). In the case of freshly isolated protoplasts *Beta* was approximately 30 % more susceptible than *Hordeum* following 3h irradiation, with viability decreasing from 90 % to 40 %. After storage of protoplasts on ice for 16 h UV-B radiation markedly depressed viability in both species, but in the case of *Hordeum* there was a substantial initial loss of nearly 70 % in viability over the first hour of irradiation.

The first 10 min of UV-B radiation decreased the intensity of DLE by 40 % without appreciably affecting the decay rate. Longer treatment times did not give a proportional effect so that even after 60 min of UV-B the inhibition did not exceed 60 %. This suggested that although the enzyme system responsible for FDA hydrolysis may be partially inactivated (viability was 75–80 % as compared with 90 % in the control), the UV-B did not penetrate the innermost parts of the chloroplasts, but left some thylakoids undamaged.

Key words: Beta vulgaris, Hordeum vulgare, protoplasts, ultraviolet radiation, viability, delayed light emission, fluorescence.

Introduction

Interest in the biological consequences resulting from an increase in ultraviolet radiation levels has arisen from fears that human activity is contributing to a decreased ozone layer, the principal absorber of UV radiation. With a decreased stratospheric ozone content, more solar UV radiation in the UV-B range of 290–320 nm will penetrate to the biosphere. Since UV-B radiation effects are mostly damaging to living systems, an increase in the level of UV-B could conceivably lead to damage to plants. Thus in an attempt to better assess the potential damage to plant cells a study was carried out on two economically important crop plants, *Beta vulgaris* and *Hordeum vulgare*. To avoid complicating factors due to a radiation gradient within can-

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Abbreviations: FDA, 3', 6'-diacetyl fluorescein; DLE, delayed light emission; UV-B, ultraviolet-B.

opies and tissues, protoplasts, as a first step, were isolated from these two plants and their viability and photosynthetic response investigated after irradiation with UV-B.

Materials and Methods

Plant material and culture conditions

Seeds of sugar beet (*Beta vulgaris* L., cv Primahill derivative 9164) were germinated and the seedlings grown in vermiculite with nutrient irrigation in a greenhouse with supplementary continuous illumination (Osram Power Star HQI-R and Tungsram incandescent, 38 W m^{-2}). Temperature was ca. 22 °C. Barley seeds (*Hordeum vulgare* L., cv Simba) were germinated and cultivated in a growth chamber in sand and watered as required with half-strength nutrient medium. Illumination was from General Electric Power Groove F48 (17 W m^{-2}) and temperatures were 23 ± 2 °C (day) and 17 ± 2 °C (night).

Barley was harvested when 7 and sugar beet when 21 days old. Prior to use sugar beet was kept in the dark for 12 h in an attempt to reduce the starch level in the leaves. Leaves were disinfested by sequential immersion in 70 % ethanol (10 s) and 20 % commercial bleach (Klorin, 10 min) and the residual sterilant removed by three sterile distilled water rinses. The abaxial epidermis was stripped off the two most fully expanded sugar beet leaves and the exposed parts cut into ca. 15–20 mm^2 segments. The segments were preplasmolyzed by floating their exposed surfaces on 15 ml of sterile rinsing medium in 90 mm plastic petri dishes siliconized with Sigmacote. Barley leaves, of which the apical and basal 10 mm were discarded, were sliced into 1 × 1 mm segments with the ad- and abaxial epidermis left intact, and transferred to 15 ml of isolation medium as above. The amount of leaf tissue used per ml of isolation medium was ca. 100 mg sugar beet and 175 mg barley.

Isolation and culture media

The isolation media used were, with slight modifications, virtually the same as those reported by Edwards et al. (1978): (1) Medium 1, a rinse and isolation medium consisting of 0.5 M sorbitol, 1 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, pH 5.5; (2) Medium 2, a flotation medium, 0.5 M sucrose, 1 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, pH 5.5; and (3) Medium 3, a collection medium, 0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, pH 5.5. Enzymes were dissolved in Medium 1 and consisted of either 0.3 % Macerozyme R-10 and 2 % Cellulase Onozuka R-10 (both from Yakult Honsha) or 0.1 % Pectolyase (Seishin Pharmaceutical) and 2 % Cellulase Onozuka R-10. After dissolving in Medium 1 the enzyme solutions were centrifuged at 1000 g for 10 min, filtered through a series of three Munktell filters (Nos. 3 + 1F + 00 in that order) and finally sterilized through a 0.2 µm membrane filter using a nitrogen-charged dispensing pressure vessel. Following purification, protoplasts were transferred to and kept on ice until required in a sterile Murashige & Skoog (1962) culture medium, one-tenth strength with respect to macro- and microelements and 0.4 M with respect to sorbitol. The medium lacked plant growth substances.

Protoplast isolation

The petri dishes containing the leaf segments were drained of rinse medium using a 10 ml pipette, the tip covered by a piece of 120 µm mesh nylon bolting cloth. Fifteen ml enzyme solution were immediately pipetted into the individual petri dishes, resuspending the leaf segments. The dishes were then placed in an orbital incubator shaker at 30 ± 1 °C and left to incubate for 1.5 to 3 h in the dark.

At the end of the incubation period the enzyme medium was removed by gentle aspiration with a pipette covered by a 120 µm mesh filter and discarded. (The spent medium contains very

few protoplasts.) The remaining leaf segments were then resuspended in 10 ml aliquots of Medium 1 and shaken gently for 1 to 2 min to dislodge and separate the protoplasts from the partially undigested tissue. The liquid containing the released protoplasts was then consecutively filtered through 120 and 74 µm (sugar beet) and 120 and 60 µm (barley) mesh filters. The washing procedure was repeated thrice more and, after combining, the filtrate was centrifuged at 100 g for 5 min. The supernatant was discarded and the protoplast pellet gently resuspended in a few drops of flotation medium (Medium 2). The resuspended protoplasts were then poured into modified 6 ml glass centrifuge tubes. This tube (Fig. 1) with its narrow, graduated neck, facilitates the concentration and recovery of pure, intact protoplasts and has the advantage over the 50 ml Babcock bottle (Shepard and Totten, 1975) in that a fraction of the flotation media are required. The protoplast suspension in each collection tube was made up to 4 ml with Medium 2 and then carefully overlayed with 1 ml Medium 3. Finally, a few drops of Medium 1 were added (Edwards et al., 1978). After centrifugation at 50 g for 3 to 5 min, the layer of purified protoplasts at the interface of Media 3 and 1 was collected and transferred to the culture medium.

Viability tests and fluorescence microscopy

Commonly used substances for assessing viability of intact protoplasts are the fluorescence reagents fluorescein diacetate (FDA) and fluorescein isothiocyanate, and for damage or death, exclusion dyes such as methylene blue, Evan's blue, bromphenol blue and pherosafranine. Of these phenosafranine seems to be a reliable indicator for nonviable or dead cells and the fluorescence dyes for viable or living cells (Widholm, 1972; Galbraith and Mauch, 1979; Brunius, 1980). Other techniques such as protoplasmic streaming and plasmolysis have also been employed to estimate the relative viability of plant cells. However, the most convenient and reliable method has been that of vital staining. The staining mechanism is based on the cleavage by endogenous esterases of the FDA molecule with the subsequent release of fluorescein, an hydrolytic polar product of FDA. These freed molecules fluoresce, the accumulation of observable fluorescence usually requiring 1 – 5 min.

In most of the brightfield, phase, interference contrast and fluorescence observations, use was made of precision rectangular glass capillary tubes (Microslides, Vitro Dynamics) with a pathlength of 200 µm. Viability was estimated visually with 0.01 % FDA. Fluorescence was examined with an Olympus BH-RFL fluorescence microscope incorporating dichroic Ploem mirrors. Exciter filter BG-12, dichroic mirror B and barrier filter O-530 were used. Photographs were recorded on either Agfachrome 50 L, Agfapan Vario-XL (125-1600 ASA) or Ektachrome (400 ASA daylight and 160 ASA Tungsten). Viability was scored as viable (brilliant-white to green fluorescence), semi-viable (yellow to orange) and non-viable (red). Unstained, living protoplasts showed a red autofluorescence. The flat capillary tubes greatly facilitated viewing and counting. Twenty separate counts, each involving up to 50 protoplasts, were made of each replicate. This procedure was carried out in respect of freshly isolated and 16-hour-old protoplasts following dark and UV-B treatments. Three replicates were used per experiment. Experiments using separate sets of plants were repeated at least twice, most frequently three to four times. Samples were also taken for determining dimensions and density (Bürker haemocytometer).

In conjunction with FDA tests a method for measuring delayed light emission (DLE) was used as an estimate of potential UV-B radiation damage specifically to the photosynthetic system. This was done in an attempt to obtain a more accurate, non-subjective measurement.

Delayed light emission

This results from the reversion of reactions involving the conversion of light energy to chemical energy in the photosynthetic system. Stored chemical energy is re-emitted as light which can be measured by a suitably sensitive apparatus. Following the discovery of DLE by Strehler

and Arnold (1951), the close connection to the photosynthetic process has been established by studying the influence of factors which also affect the photosynthetic system (Lavorel, 1975).

The light emission from the protoplasts was measured with a sensitive light meter. The light to be measured is modulated by a chopper disc at a frequency of 35 Hz. The AC-signal from the photomultiplier (type HTV R374) is amplified in a timed amplifier and phase-locked to the light signal by a second chopper disc between a lamp and a photodiode. The signal is then integrated and fed to a recorder.

Preparation for dark and UV treatments

Using a pasteur pipette, four drops (ca. 20 µl) of either sugar beet or barley protoplast suspension were positioned on the base of siliconized 50-mm plastic petri dishes with two drops of nutrient medium on the inside surface of the fitted lid in order to help maintain humidity in the dish when under irradiation. The base of the dish was then carefully inverted on to the lid, fitted and sealed with Nescofilm and placed 120 mm from preburnt FS 20 sunlamps (Westinghouse; 22 W m^{-2}) under aged 0.1-mm-thick cellulose acetate filters cutting off radiation below 290 nm. Protoplast samples were irradiated for 1, 2 or 3 h. Control samples were placed in darkness and the viability tested with FDA at the start and termination of the experiment. The procedure commenced after the protoplasts had stabilized for 0.5 h in the dark and was later repeated on protoplasts held overnight for 16 h in culture medium on ice.

Preparation for delayed light emission

Isolated protoplasts of barley and sugar beet were transferred immediately after isolation into 12 ml of culture medium, placed on ice and left to stabilize in the dark for 0.5 h. Two ml of protoplast suspension were then pipetted into six individual siliconized 50-mm plastic petri dishes which included two dark controls, one at the onset of the experiment, and the other at its termination, and three light treatments under the same FS 20 sunlamp and filter systems as described above, with irradiation times of 10, 30 and 60 min. During the irradiation periods the dishes were gently shaken periodically to ensure that all protoplasts were evenly irradiated from all directions. After each irradiation treatment the protoplasts were allowed to rest in the dark for 15 min before DLE was measured. The treated or untreated (dark control) protoplasts were pipetted into the 850-µl plexiglass cuvette of the DLE meter. The cuvette was inserted into the apparatus and the sample irradiated with far-red light (200 W m^{-2}) using a 2-mm glass filter, type RG 715 (Schott), for exactly 1 min. The light was then switched off and the DLE signals were relayed to a recorder. Readings were made at 0.5 min intervals up to 4 min. The procedure was the same for both sugar beet and barley and was repeated at least twice. In order to procure consistently measurable readings at least ca. 0.7 to 1.0 × 10^5 protoplasts were required in the cuvette, meaning an initial harvest of up to 1.5 × 10^6 purified protoplasts per ml.

Results and Discussion

Results from the microscopic analysis of protoplast dimensions for barley and sugar beet are given in Table 1. The wide range in size (Fig. 2) of sugar beet protoplasts (Li-Su-Nam et al., 1976) in large measure reflects the bifacial nature of this plant's leaf, but is probably also due to the fact that spontaneous fusions may have occurred, accounting for diameters considerably greater than were screened by the 74-µm-mesh filter. Predictably, the coefficient of variation is large, three times that of barley. Not only are the barley protoplasts (Fig. 3) uniformly smaller – about one-half the size of those of sugar beet – but they also have nearly one-fourth the proto-

Table 1: Diameters (µm) of mesophyll protoplasts of one- and three-week-old *Hordeum vulgare* and *Beta vulgaris* respectively, as well as mean area ratios of protoplast : nucleus. s, standard deviation; CV, coefficient of variation.

	Hordeum	*Beta*
Range (min – *mean* – max)	22.5–*30.5*–42.5	25.0–*51.9*–120.1
Mode	35.0	37.5
s	4.2	21.2
CV, %	13.6	40.8
Mean area, ratio, protoplast : nucleus	7.8 : 1	27.6 : 1

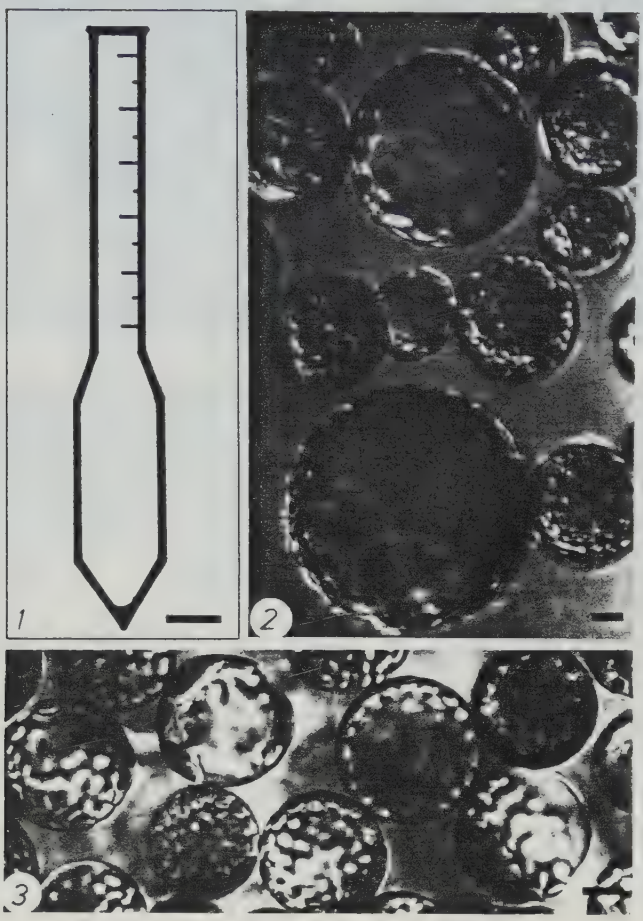
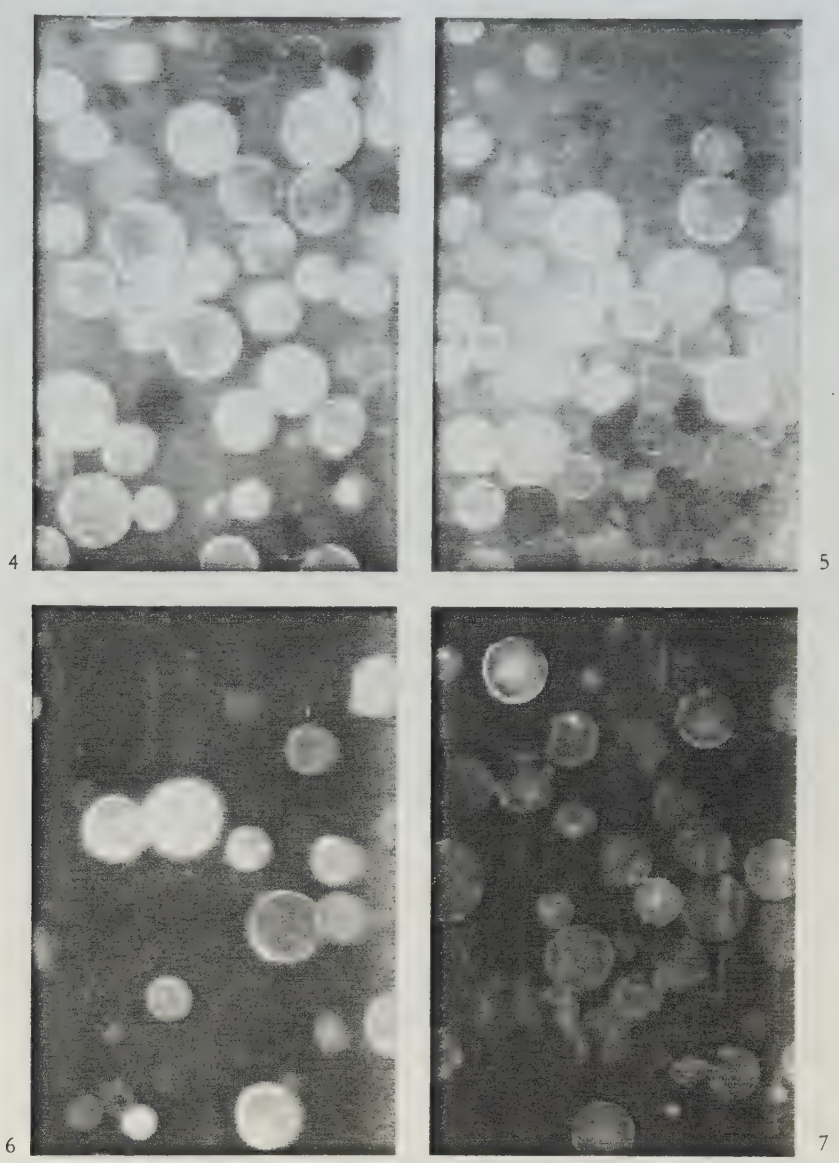


Fig. 1: Specially-constructed 6 ml centrifuge tube for the collection of pure, intact protoplasts by flotation. Scale bar = 10 mm.

Fig. 2: Interference contrast micrograph of *Beta vulgaris* mesophyll protoplasts. Scale bar = 10 µm.

Fig. 3: Interference contrast micrograph of *Hordeum vulgare* mesophyll protoplasts. Scale bar = 10 µm.



Figs. 4–7: *Beta vulgaris* mesophyll protoplasts stained for 5 min with 0.01 % FDA in culture medium (0.4 M with respect to sorbitol) and photographed with Ektachrome 160 ASA (tungsten) film. – Figs. 4 and 5 Non-irradiated protoplasts, freshly-isolated (0.5 h) and aged for 16 h, respectively. – Figs. 6 and 7 Freshly-isolated and aged protoplasts irradiated with UV-B for 3 h; accumulated age at time of exposure 3.5 h (Fig. 6) and 19 h (Fig. 7).

plast: nucleus surface area ratio of the latter, presumably indicating a greater potential stability.

Figures 4–7 illustrate some of the visual effects of UV-B radiation damage to sugar beet protoplasts. Figures 4 and 5 are dark controls of freshly-isolated and 16-h-old protoplasts (approximately corresponding to the material represented by the solid- and open-starred arrows in Fig. 8) and indicating a more than 25 % decrease in viability over the course of two-thirds of a day, despite being stored on ice. Figures 6 and 7 show the effects of 3 h of irradiation with UV-B; the freshly-isolated protoplasts in

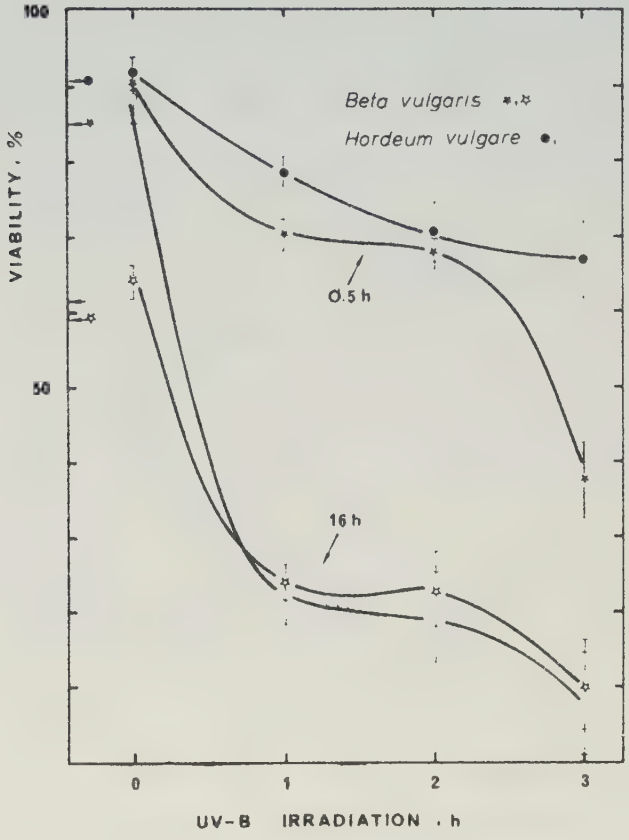


Fig. 8: Effect of UV-B radiation on viability of *Beta vulgaris* (★, ☆) and *Hordeum vulgare* (●,) mesophyll protoplasts as measured by FDA vital staining. Protoplasts were used either one-half hour or 16 h after isolation. Viable protoplasts were scored as those that fluoresced brilliant-white to green and showed no intracellular clumping of particulate matter. Arrows on the ordinate indicate percentage viability of dark controls at the termination of the experiments, 3 h (upper set) and 19 h (lower). Vertical bars indicate standard deviation.

Fig. 6 displaying early signs of metabolic damage by fluorescing yellow to orange, and the aged in Fig. 7 a nearly 90 % non-viability characterized by a red fluorescence.

The results were also expressed as percentage viability (Fig. 8). Of the protoplasts that were treated with UV-B radiation, there was an appreciable difference in percentage viability between those used 0.5 h or 16 h after isolation. As far as differences between the two species are concerned, freshly-isolated sugar beet protoplasts appear the more susceptible, showing a greater loss of viability with time after irradiation as compared to barley, the difference – nearly 30 % – being most marked after 3 h of UV-B treatment. The viability of untreated protoplasts remained relatively stable during the time course of the experiments. This is shown in Fig. 8 by the four arrows along the ordinate which give the percentage viability at the termination of the experiment, that is at 3.5 and 19 h respectively. In the untreated protoplasts, barley had still maintained a high proportion of viability, whereas that of sugar beet

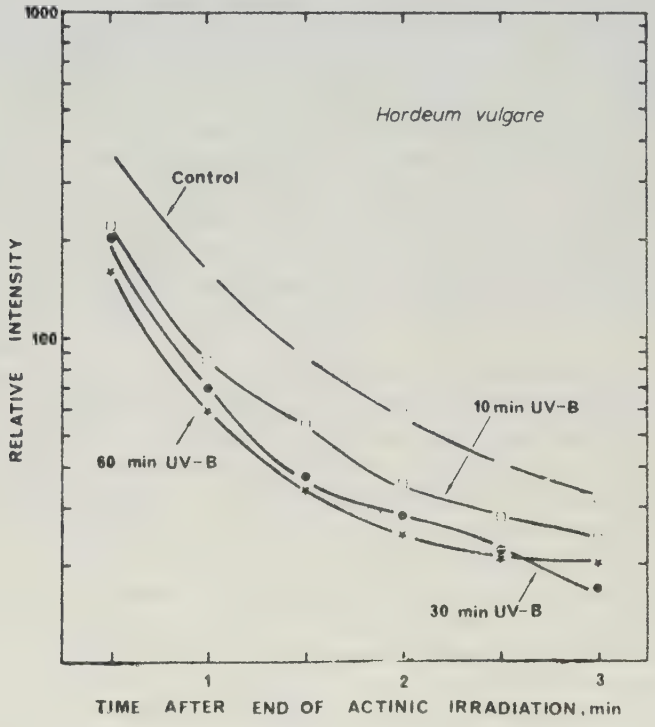


Fig. 9: Intensity (logarithmic scale) as a function of time in *Hordeum vulgare* mesophyll protoplasts. Delayed light emission (DLE) was recorded at time zero as well as 10, 30 and 60 min after irradiation with UV-B on freshly-isolated protoplasts. ○, control; □, 10 min; ●, 30 min; ★, 60 min.

was slightly lower. However, upon irradiation with UV-B, more or less the same trend was followed by both barley and sugar beet. This was not the case in the freshly isolated material after treatment with UV-B had reached 3 h. After 3 h irradiation, namely, freshly isolated sugar beet protoplasts were 20 % less viable as compared to those left untreated but aged for 16 h. Only those protoplasts judged as fully viable, that is fluorescing brilliant-white to green, were used in the data shown in Fig. 8.

Although it would appear that after 16 h barley is the more sensitive of the two to irradiation, variables such as handling of the material, subjective estimates of viability, as well as inherent protoplast robustness have to be taken account of. For example, very large sugar beet protoplasts tend to be unstable and burst more easily, thus avoiding detection with FDA. The general similarity in response of both sugar beet and barley (Fig. 8) used 16 h after isolation is probably due to the levelling out of differences as a result of the advanced age of the protoplasts coupled with the effect of the UV-B treatment.

Figure 9 shows the DLE results obtained in respect of barley. The first 10 min of UV-B treatment decreases the intensity of DLE by about 40 % without appreciably changing the decay rate. Longer times of irradiation, however, do not give a proportional effect, and even after 60 min of UV-B the inhibition does not exceed 60 %. Two explanations for this are possible: either (1) the UV-B does not penetrate to the innermost parts of the chloroplasts so that some thylakoids remain undamaged or, (2) there are two different components of DLE, only one of which is affected by UV-B radiation. Of these (1) appears more likely, since the time course of the emission is not changed, and it would be a strange coincidence if there were two emission components with the same decay rate. Sugar beet protoplasts, although displaying a similar rate of decay as barley, actually showed a slightly greater decrease in the intensity of DLE.

It should be stressed that even though conditions may be rigorously standardized, caution should be exercised in the interpretation of the fluorescent image. Thus not only are concentrations and freshness of both dye and protoplasts and time of staining critical, but also the photographic exposure should be kept as short as possible since progressive fading and blurring presumably as a result of damage by UV light from the microscope's mercury burner occur with time. In addition, with the combination of filters used, the leaf mesophyll protoplasts give a red autofluorescence in unstained preparations. This also applies – for a period – to chloroplasts released from freshly-broken protoplasts, making adequate controls essential. Furthermore, as is evident from Fig. 7, it was frequently observed that fluorescence in UV-treated, FDA-stained material was limited to only a portion of what appeared to be an intact protoplast, possibly indicating leakage as a result of membrane damage or localized inhibition of the esterases involved in hydrolysis of FDA to fluorescein. When freshly isolated, sugar beet protoplasts are significantly more sensitive to UV-B radiation, especially after 3 h than are those of barley. However, when stored protoplasts of both plants are irradiated the loss in viability in barley in comparison to the

control is about 70 % during the first hour, more than 1.5 times greater than in sugar beet. From our present observations it is not possible to determine whether viability and loss of chloroplast activity bear any direct relationship to one another, but Rathnam and Edwards (1976) could store *Nicotiana* protoplasts in the cold for 20 h without appreciable loss in activity of the chloroplasts upon their isolation.

It should be pointed out that the term «viability» is relative, and the sole criterion in this study was the ability of a class of enzymes to carry out a specific hydrolytic reaction. Viability in terms of eventual plant regeneration from the protoplast has not been achieved with either species. The results of the fluorescence images show the loss of the protoplast's enzyme activity due both to UV-B irradiation and ageing, and as seen from the DLE results even a 10 min exposure to UV-B causes a substantial loss in photosynthetic activity (Fig. 9).

This study has dealt not only with viability methods, but has also shown some effects of ultraviolet radiation per se, as mirrored in these viability tests. It is hoped that these methods of assessing damage can be used in further work involving the effects of UV radiation on plants.

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The Effect of UV-B and UV-C Radiation on Sugar Beet Leaves

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Summary

The effect of UV-C (254 nm) and UV-B (290 320 nm) radiation on leaves of *Beta vulgaris* L. at the ultrastructural level was investigated. Although the damage caused by UV-C radiation was more striking than that resulting from UV-B radiation, several structural changes were seen in the UV-B treated material. Generally the effects of UV-B and UV-C radiation were different, suggesting different mechanisms of action, discernible even at the ultrastructural level.

Keywords: *Beta vulgaris*; Chloroplast; Ultrastructure; Ultraviolet radiation.

1. Introduction

The ozone layer, an effective absorber of most of the ultraviolet (UV) radiation, is constantly being modified by both the addition of ozone molecules (O_3) and their chemically catalyzed dissociation. It follows that any addition of chemical catalysts which destroy O_3 (*e.g.*, from the photodissociation of chlorofluoromethanes, used mainly in spray propellants and as refrigerants; nitric oxides from supersonic aircraft, nuclear bombs, excess use of nitrogen fertilizers, etc.) will result in an increase of damaging UV radiation reaching earth.

Although UV radiation makes up only ca 7% of the solar radiation reaching the earth's surface, it is biologically important because of its effective absorption by biological macromolecules and high energy content per photon that can result in a range of photochemical reactions both advantageous and disadvantageous (CALDWELL 1981). Different wavelengths elicit different responses. The effects of UV-C (< 290 nm) and probably also UV-B (290 320 nm) radiation are generally harmful to the plant system.

Early experiments on the effects of UV radiation were carried out mainly with UV-C which is readily obtained from 15 W germicidal lamps, giving radiation almost exclusively at 254 nm. Nucleic acids absorb very effectively at this wavelength, and much research has been done on the inactivation of these acids. UV-B radiation can also induce photobiological changes in nucleic acids, although it is less effective (HARM 1979). The inhibition of photosynthesis, especially by UV-C, has been rather thoroughly investigated, although the site of UV photoinhibition has not been conclusively established (MANTAI and BISHOP 1967, MANTAI *et al.* 1970). Ultrastructural effects of UV radiation have, on the other hand, not been as well studied and there is a general lack of reliable information, especially concerning the effects of UV-B radiation, the consequences of which are much more difficult to detect than those of UV-C radiation.

This study was prompted by an interest in the possible relationship between physiological effects and changes at the ultrastructural level. We were interested in ascertaining whether physiological damage is necessarily paralleled by structural changes. If this is the case, what is the nature of these changes, can they be detected

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at the electron microscope level, and what might they imply at the individual cell level and for the plant as a whole? Previous results (BORNMAN *et al.* 1982) from delayed light emission studies on the photosynthetic system suggested that UV-B radiation was not penetrating to the innermost parts of the chloroplasts, resulting in some thylakoids remaining undamaged. This was confirmed in the present study, with a variation in damage not only at the chloroplast level, but also among cells. This would conceivably result in non-proportional effects of UV radiation damage at the physiological level (BORNMAN *et al.* 1982).

Although UV-C radiation, because of its absorption by the ozone layer, does not penetrate to the earth in any appreciable amount, it was included in the study as a possible indicator of or clue to damage resulting from the longer wavelengths of UV-B. Nevertheless the distinct possibility of UV-B and UV-C radiation causing different structural effects was not ruled out, since at the level of photosynthesis UV-B radiation appears to have its effect mainly on photosystem II (BRANDLE *et al.* 1977, JONES and KOK 1966, NOORUDEEN and KULANDAIVELU 1982). The question as to whether these differences are mirrored structurally may also depend on penetration or absorption differences between UV-B and UV-C radiation of the tissue. For this reason results obtained from UV-B and UV-C should not be used to substantiate each other, as was done by BRANDLE *et al.* (1977).

2. Materials and Methods

Seeds of *Beta vulgaris* L. (cv. Primahill, derivative 9164) were grown in vermiculite in a growth chamber (illumination was supplied by General Electric Power Groove F 48, 17 W m $^{-2}$; 16/8 hours day/night regime; day temperature, 23 ± 2 °C and night, 17 ± 2 °C).

When the seedlings were two weeks old, they were grown for a further 7 days with illumination from either General Electric Power Groove (controls), or supplemented with UV-B radiation from FS 20 and FS 40 sunlamps (Westinghouse; 9.46×10^{-2} W m $^{-2}$), which were filtered through aged 0.1 mm thick cellulose acetate, effectively cutting off radiation below 290 nm. The controls and treated material were kept separate by a 6-mm-thick Plexiglas sheet that completely screened off the ultraviolet radiation from the control section. The treated plants were illuminated in the middle of the light period with the supplemental UV-B for 6 hours within a 24 hour cycle.

The procedure was similar for UV-C treated plants, except that irradiation from a 254 nm low-pressure mercury lamp (1.63 W m $^{-2}$) was for only 1.5 hours within a 24 hour cycle. Both the UV-B and UV-C irradiated plants, together with the controls, were prepared for electron microscopy after the pre-determined treatment periods were over. The plants were harvested after the dark period.

Leaf segments (2 × 1 mm) were cut with sharp razor blades in 0.05 M cacodylate buffer. Segments were taken equidistant between the margin and midvein from leaves of similar age. The segments were fixed in 6% glutaraldehyde and postfixed in 2% osmium tetroxide. The material was then dehydrated in a graded ethyl alcohol series and propylene oxide before being embedded in Agar 100 Resin-Araldite (Agar Aids, Essex, England). Thin sections were cut with a diamond knife using a Sorvall MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed and photographed with a Hitachi HU-11C microscope. Ten replicate samples of leaf segments from control as well as from treated material were used for sectioning. Three hundred and forty-seven electron micrographs were made of control and treated material, and the actual number of sections examined under the electron microscope numbered from 500 to 1000 each for control and treated material.

3. Results and Discussion

3.1. Control Tissue

Leaf material from control plants was characterized by well-defined cell structure (Fig. 1). The highly vacuolate mesophyll cells contained numerous chloroplasts with well-developed grana and stroma thylakoids. Plastoglobuli of variable size were also present in the finely granular stroma. Few starch grains were to be found, although many electron lucent areas in the stroma between thylakoids indicated the apparent location of recently mobilized starch. Fibrils of DNA were not discernible in such areas. The nucleate mesophyll cells also contained a large population of mitochondria and microbodies. Some dictyosomes, variable numbers of paramural bodies (plasmalemmasomes), and abundant tubular endoplasmic reticulum (ER) were present. Ribosomes were associated with the ER and unattached in the cytoplasm.

3.2. Effects of UV-C Radiation

Of the two treatments, leaf tissues exposed to UV-C showed the greatest damage. Large portions of the adaxial surfaces of the leaves were extensively damaged, as reflected in the collapse and near obliteration of epidermal cells and underlying palisade parenchyma (Figs. 2–4). In Fig. 2, the contents of the epidermal and of the palisade parenchyma cells on the left are little more than an amorphous mass. In two of the palisade cells (middle and right), remnants of the chloroplasts are still discernible in the parietal layer of cytoplasm.

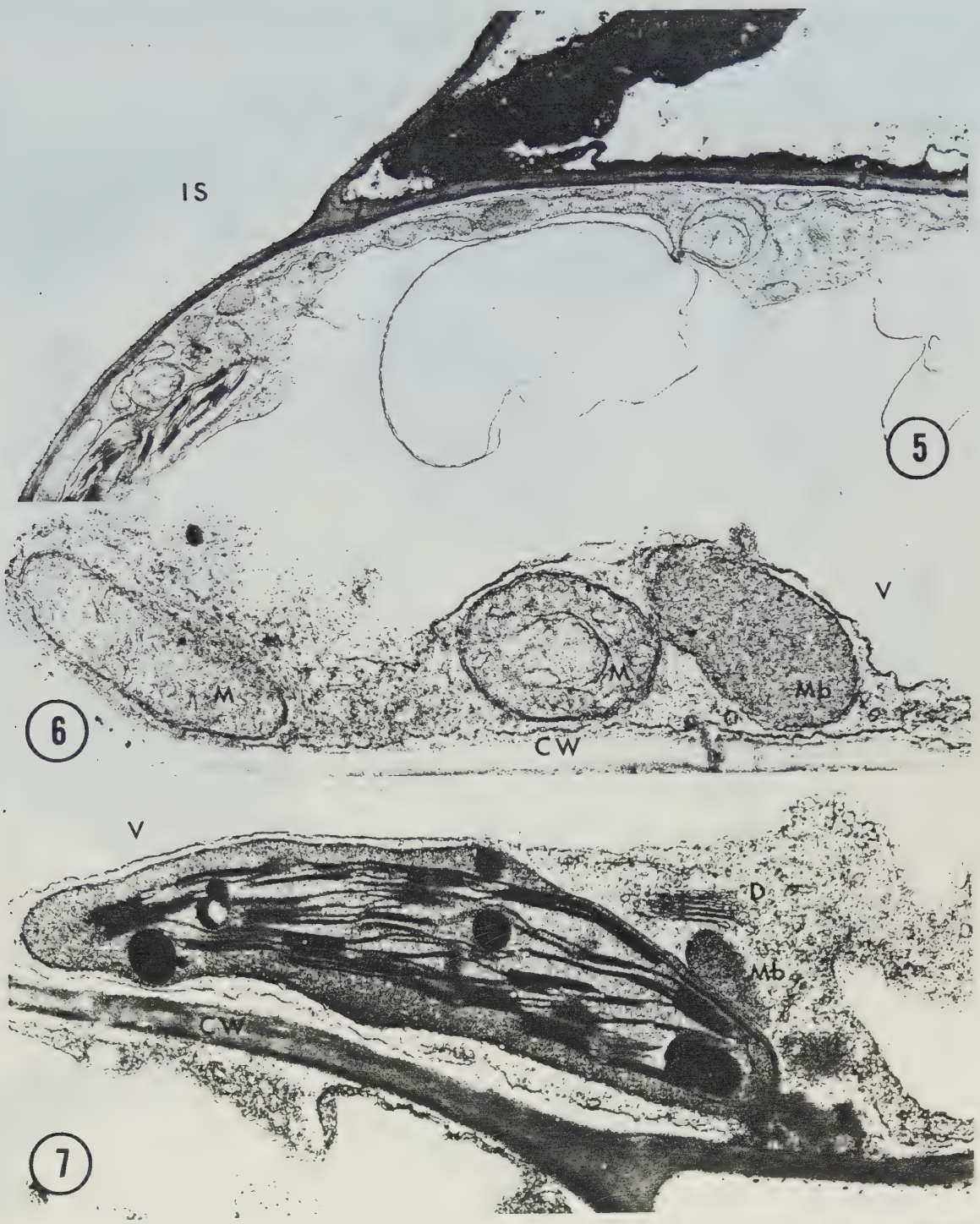
Another result of UV-C treatment is illustrated in Figs. 3 and 4, namely, the presence of relatively large numbers of starch grains in the severely damaged palisade parenchyma. In the collapsed cells of Fig. 3 the starch-containing chloroplasts are no longer seen as discrete entities, whereas in the partially collapsed

Fig. 1. Portion of mesophyll cell from control tissue. *CW* cell wall, *IS* intercellular space, *M* mitochondrion, *N* nucleus, *V* vacuole. Unlabeled arrows point to electron lucent areas in stroma of chloroplasts that apparently correspond to sites once occupied by starch grains. × 12,200

palisade cell of Fig. 4 the starch grains are clearly visible within the discrete, although denatured, plastids. The extent of starch content exhibited by some cells of the UV-C treated tissues was not encountered in either UV-B treated or control tissues, and obviously is a reflection of the inability of such severely damaged cells to mobilize starch. This immobilization could be due to either membrane damage, resulting in a loss of the permeability properties, or to destruction of enzymes.

A considerable range in degree of structural damage was found in the UV-C treated leaves—from total obliteration to no apparent damage whatever. As illustrated in Fig. 5, cells of normal appearance commonly were contiguous to badly damaged ones. The most severely damaged cells were located in or near

Figs. 2-4. Adaxial portions of leaves exposed to UV-C radiation showing extensive damage to epidermal cells and underlying palisade parenchyma. Fig. 2. Nearly obliterated epidermal cells (above) and partially collapsed palisade parenchyma (below). *C* chloroplast, *IS* intercellular space. × 5,500. Fig. 3. Collapsed palisade parenchyma cells. The numerous clear, ovoid areas within the mostly amorphous protoplasts are starch grains. *IS* intercellular space. × 3,200. Fig. 4. Partially collapsed palisade parenchyma cells in which the denatured, starch-containing chloroplasts are still discernible in the parietal layer of cytoplasm. *V* vacuole. × 4,500



Figs. 5 7. Portions of mesophyll cells from leaves exposed to UV-C radiation showing variability in degree of ensuing structural damage. Fig. 5. Severely damaged cell (above) bordering cell of normal appearance (below). *IS* intercellular space. × 11,500. Fig. 6. Detail of portions of apparently healthy cell that bordered on severely damaged cell. *CW* cell wall, *M* mitochondrion, *Mb* microbody, *V* vacuole. × 40,000. Fig. 7. Chloroplast in which fusion of both grana and stroma thylakoids has taken place. *CW* cell wall, *D* dictyosome, *Mb* microbody, *V* vacuole. × 28,600

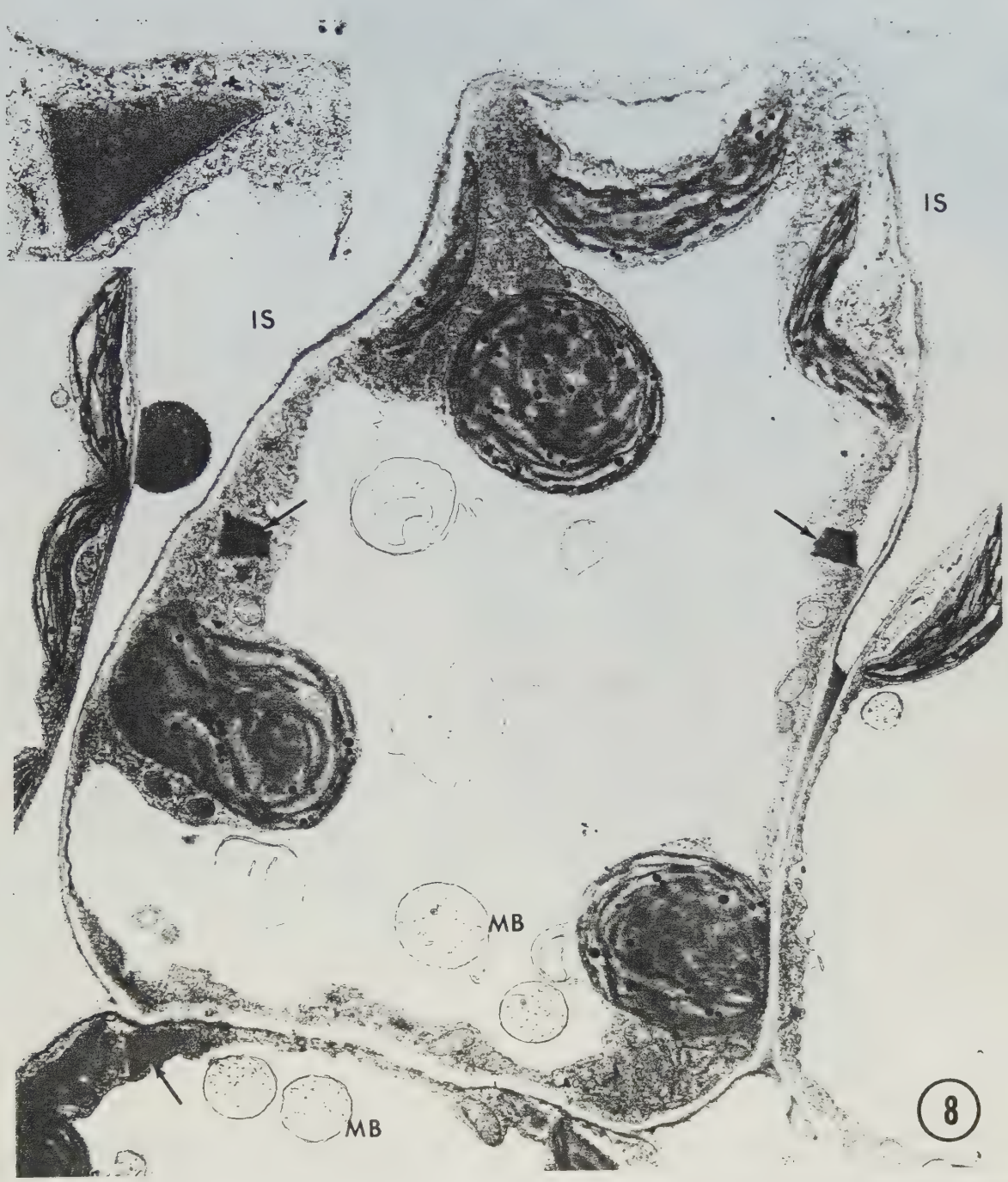


Fig. 8. Portions of four mesophyll cells from leaf exposed to UV-C radiation, showing membrane-bound crystalloids (unlabeled arrows and inset) found in many cells after this treatment. The multivesicular bodies (*MB*) in the large, central vacuoles of these cells are derived from paramural bodies. They also are found in cells of control tissues. *IS* intercellular space. × 6,400. Inset × 34,000

the adaxial surface of the leaf, with decreasing damage correlated with increasing distance from the surface. This gradient may be explained by a number of factors such as absorption of the UV radiation by phenolic compounds (WELLMANN 1971, ROBBERECHT and CALDWELL 1980) and other cellular components. Fig. 6 shows a portion of an apparently "healthy" cell that was contiguous to a severely damaged one.

Among the various cellular components, the chloroplasts appear to be most sensitive to UV-C radiation (see MANTAI *et al.* 1970). One of the earliest symptoms of treatment with UV-C radiation is the loss of integrity

Fig. 9. Portions of two mesophyll cells representative of most such cells after exposure of leaf to UV-B radiation. *C* chloroplast, *CW* cell wall, *IS* intercellular space, *M* mitochondrion, *N* nucleus, *V* vacuole. ×22,200

of the thylakoids as they undergo adpression (fusion). This phenomenon is illustrated by the chloroplast in Fig. 7. Although obliqueness of a section might result in a similar image, in this case it was clearly membrane fusion resulting from the UV-C treatment. A great many sections, including serial sections, were examined of such chloroplasts to confirm this interpretation. Another symptom of UV-C radiation is the appearance of membrane-bound crystalloids in the cytoplasm of many of the cells, which otherwise show no other effects. Three such crystalloids can be seen in the mesophyll cells of Fig. 8. The crystalloid at the lower

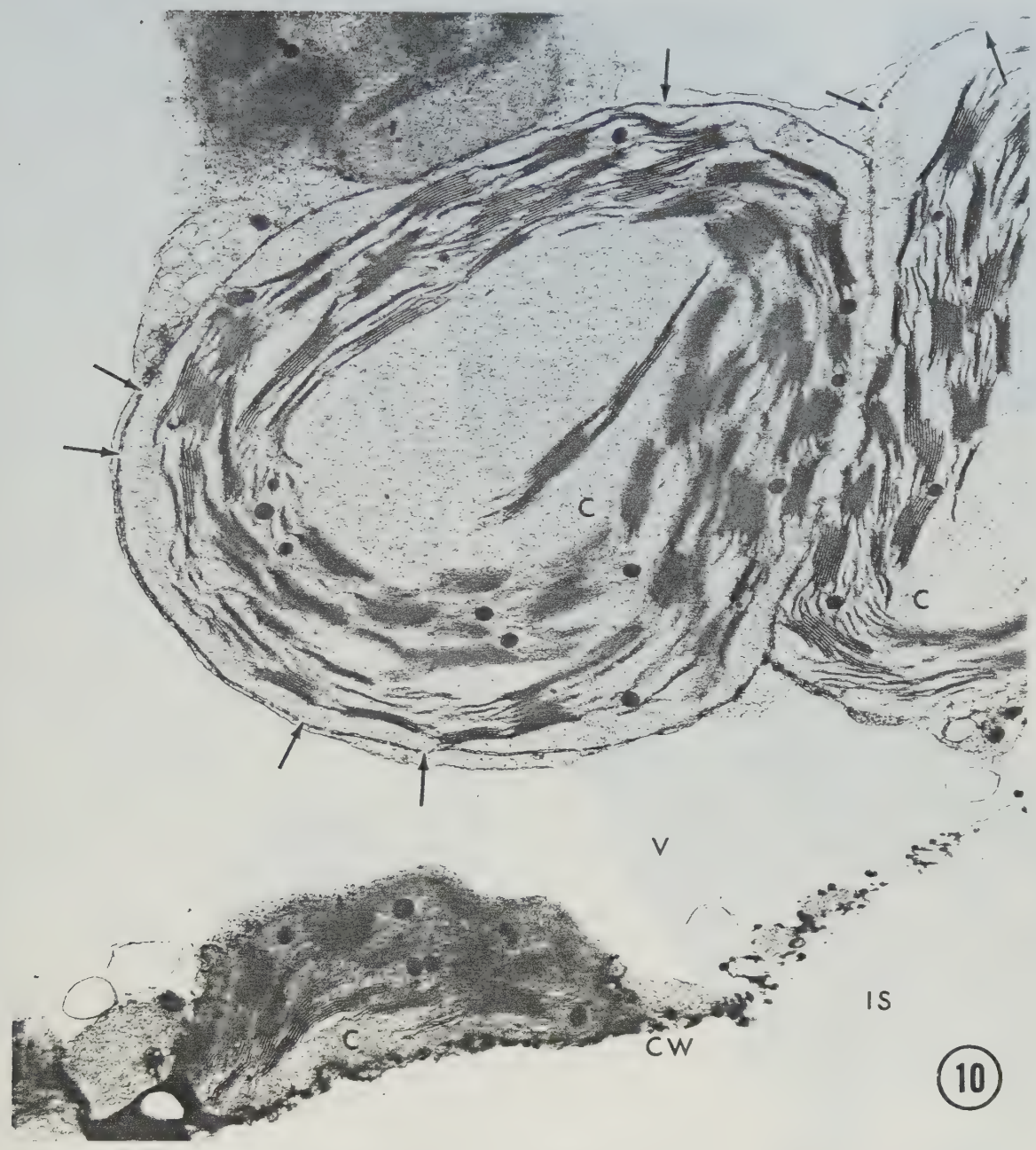


Fig. 10. Portion of mesophyll cell from leaf exposed to UV-B radiation. Unlabeled arrows point to disruptions in the chloroplast envelope, a condition apparently resulting from the treatment. *C* chloroplast, *CW* cell wall, *IS* intercellular space, *V* vacuole. ×22,700

left is shown at greater magnification in the inset. The bounding membrane is distinct along only one surface; it is sectioned obliquely along the other two. The site of origin of such crystalloids remains to be determined; two candidates are dilated ER cisternae and microbodies.

3.3. Effects of UV-B Radiation

Except in a few instances, the effects of UV-B radiation were much more subtle than those of UV-C. Fig. 9 is a representative view of the great majority of mesophyll cells exposed to UV-B. All of the cellular components seen here are normal in appearance. Nuclear pores can be seen in surface view to the left in the clearly-defined nuclear envelope. Neither the mitochondria nor the chloroplasts show any evidence of damage.

In contrast to this general picture of structural integrity, some evidence was found where damage from UV-B radiation had obviously occurred. In Fig. 10, for example, distinct disruptions can be seen in the

Fig. 11. Portions of two mesophyll cells from leaf exposed to UV-B radiation. Whereas the cell on the right was damaged, that on the left was normal in appearance. The stroma of the chloroplasts in the damaged cell has assumed a quasi-crystalline appearance (detail in inset). *CW* cell wall, *IS* intercellular space, *M* mitochondrion, *V* vacuole. ×30,000. Inset ×52,500

envelopes of two chloroplasts. In the lower chloroplast some thylakoids are dilated, and membrane degradation (the electron dense substance) is apparent in the layer of cytoplasm bordering the cell wall. Conspicuous damage to other cellular organelles appeared to be absent although there have been reports of structural effects on other organelles (BRANDLE *et al.* 1977).

The most notable damage caused by UV-B radiation is illustrated in Fig. 11, which shows parts of two mesophyll cells. The damaged cell on the right contains portions of two or three chloroplasts, which had begun to lose their structural integrity. The orderly pattern of grana and stroma thylakoids has been lost, and some of the thylakoids appear slightly dilated. Moreover, most

of the stroma has a quasi-crystalline appearance, which is shown at higher magnification in the inset. It is possible that the crystalline conformation is the result of water stress imparted by the UV-B treatment. TEVINI *et al.* (1981) reported that UV-B irradiated plants suffered water stress. However, no evidence of incipient plasmolysis could be detected in any of the cells. Dilation of thylakoids has previously been reported as one of the effects of irradiation with UV (BRANDLE *et al.* 1977). Although some thylakoids appeared dilated in UV-B treated tissues, this feature as a separate criterion of damage must be used with caution. In most of the UV treated tissues examined during the present study, there was no evidence of thylakoid dilation.

From the results of this study it was clear that sugar beet leaves exposed to UV-C radiation showed the most pronounced structural damage, although this was not always uniform throughout the tissues. Among some of the interesting features in the UV-C treated plants were the following:

(1) a decrease in damage from the adaxial to the abaxial surface of the leaf

(2) accumulation of large quantities of starch in the chloroplasts

(3) adpression or fusion of the grana stacks

(4) cytoplasmic membrane-bound crystalloids in mesophyll cells with no apparent structural damage.

As has been well documented in the literature, the effects of UV-B radiation, on the other hand, are probably mainly at the physiological level, although a few structural changes were also found:

(1) disruption of the chloroplast envelope

(2) dilation of thylakoids

(3) membrane degradation

(4) quasi-crystalline appearance of the stroma.

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Action spectrum for inhibition by ultraviolet radiation of photosystem II activity in spinach thylakoids

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Summary

The effect of ultraviolet (UV) radiation (half-band width 10 nm) in the wavelength range 248–340 nm on chlorophyll fluorescence from a thin layer of spinach thylakoid suspension was investigated. It was found that the parameter most sensitive to UV radiation was the rise time of variable fluorescence. The increase in rise time was proportional to UV photon fluence and was used for the determination of an action spectrum. The action spectrum falls off from a maximum at ca. 275 nm towards longer wavelengths and rises from a minimum at 260 nm towards shorter wavelengths. The results also suggest that the UV inhibition is mainly on the PS II oxidizing side. Possibly damage is also inflicted to the PS II reaction center.

photosynthesis inhibition; *Spinacia oleracea*; UV-B; UV-C; UV inhibition; variable fluorescence

Introduction

Interest in the effects of ultraviolet-B (UV-B, 280–320 nm) radiation on plants has increased in recent years, since pollution of the atmosphere may cause a decrease

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max}, maximum fluorescence; F_0, initial fluorescence, F_v, variable fluorescence; PQ, plastoquinone; PS I and II, photosystems I and II; Q, quencher of fluorescence/a primary electron acceptor of PS II; UV-B, ultraviolet-B radiation (280–320 nm); UV-C, ultraviolet-C radiation (< 280 nm).

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in atmospheric ozone and an increase in the intensity of solar UV-B radiation at ground level. Plant cells have several UV-B-sensitive components; among them are components of the photosynthetic system.

Neither the site nor the precise mechanism of UV damage to the photosynthetic system has been unequivocally established, although numerous studies on the effects of UV radiation have been done (e.g., refs. 1, 2; and 3, for review article). Plastoquinone (PQ) was implicated [4], but from later work [5,6] it was concluded that PQ was not the main target, at least not for UV-C radiation. Mantai [7] tentatively suggested that damage to photosystem II (PS II) occurred near the reaction center. Others have made similar deductions [8–12]. More specifically Renger et al. [11] and Iwanzik et al. [12] suggested that the reduction in variable fluoresence (F_v) may result from PS II reaction centers being transformed into dissipative sinks for excitation energy. Structural damage to membrane systems (see, e.g., refs. 13, 14) is also likely to influence F_v.

An UV action spectrum for inhibition of photosynthesis, or a partial reaction of photosynthesis, should provide an approximation of the absorption spectrum of the most sensitive component in the system (especially if internal filtering effects are accounted for or minimized, and provided energy is not transferred to the sensitive site from a separate 'antenna'), and thus aid identification of the radiation target(s). In addition, such an action spectrum can be used for calculating and comparing biologically effective doses of natural or other polychromatic UV radiation. The fact that proteins in general absorb in the UV range of interest, makes identification of a particular component difficult; it is also highly probable that several components are damaged.

Since PS II activity is reportedly much more sensitive to UV radiation than is PS I activity, we used variable fluorescence (F_v) in order to obtain an action spectrum for UV inhibition of PS II. This indicator (F_v) has not been previously used for this purpose. Work by Ögren and Öquist [15] indicates that the variable component (F_v) of fluorescence can be employed as a measure of electron transport to Q. Their measurements differ from ours in that much weaker fluorescence excitation light was used, resulting in the fluorescence rise being rate limited by the excitation light. With the strong fluorescence excitation light which we used Q becomes nearly totally reduced, also in photoinhibited samples, and thus the amplitude of variable fluorescence is only slightly decreased, and the fluorescence rise time is a good measure of electron transport rate for PS II.

Materials and Methods

Spinach thylakoid preparation

Spinach (*Spinacia oleracea* L. cv. Viking) was grown in a temperature-controlled growth room (26/20°C light/dark and a 12 h photoperiod; illumination was from General Electric Power Groove, cool white fluorescent lamps and Osram HQI/E 400 W dysprosium lamps, together giving a photon fluence rate of 400 μmol · m^{-2} · s^{-1}) and harvested when 5.5 weeks old. Thylakoid membranes were prepared

according to Åkerlund et al. [16], except that the final pellet was resuspended in a medium containing 500 mM sucrose, 2.5 mM NaCl and 5 mM sodium phosphate buffer, pH 7.4. All procedures were carried out at 4°C. The membrane suspension was made 5% (v/v) with respect to dimethylsulfoxide, diluted with the final resuspension medium to 400 μg Chl · ml $^{-1}$ and stored in liquid nitrogen until required.

Apparatus for UV irradiation and fluorescence measurements

The apparatus used is shown schematically in Fig. 1. The basic optical unit was an Aminco-Bowman spectrophotofluorometer extensively modified. An Oriel Model 8510-2 arc lamp power supply replaced the standard power supply since the latter gave too much 100 Hz ripple for fluorescence measurements in the time scale of interest. The lamps used were either an Osram XBO 150 W/2 or the corresponding Hanovia 901-C1 xenon lamp operated at 7 A. The excitation monochromator was provided with a modified lid carrying a glass filter (Corning 4-96, 5.1 mm), which could be inserted into or removed from the light path. The filter was used only during fluorescence measurements, not during UV treatment of the sample.

A modified cuvette holder with a quartz cuvette containing 40 μl diluted thylakoid suspension (1.92 μg Chl) in a 2.0 mm thick and 3.6 mm wide layer was inserted into the sample compartment. The cuvette was held against a brass plate which was cooled by water flowing in ducts from a temperature-controlled water bath. Temperature was kept at 10 ± 0.2°C. Fluorescence was measured with excitation at 440 nm, half-band width of 30 nm (photon fluence of 90 μmol · m $^{-2}$ · s $^{-1}$). Measurements of the UV absorbance of the thylakoid suspension indicated that UV fluence could be considered constant throughout the thickness of the sample. The

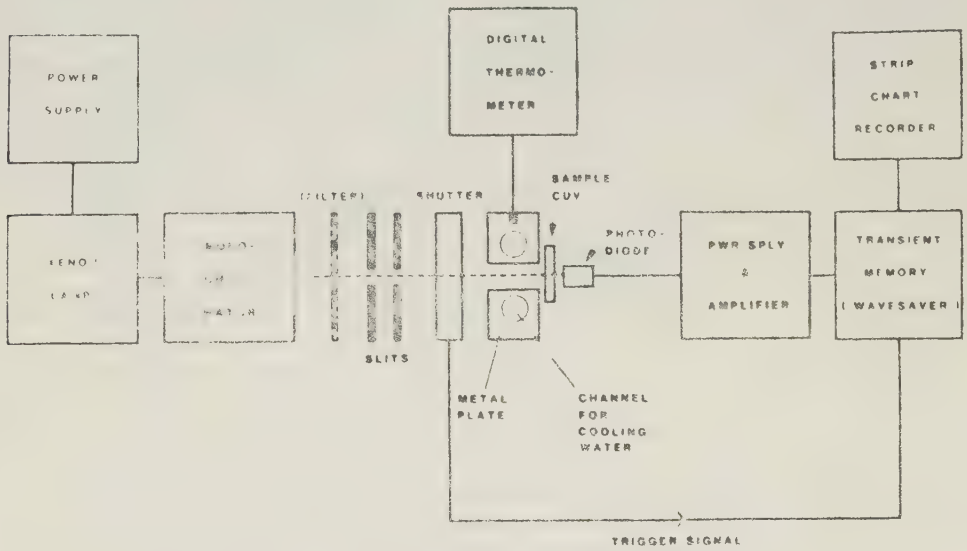

Fig. 1. Schematic representation of the apparatus for UV irradiation and fluorescence measurements.

sample was kept in the same position during both UV irradiation and fluorescence measurements.

The emission unit of the Aminco-Bowman spectrophotofluorometer was not used for fluorescence measurements (unsuitable geometry and too slow a response). Instead, detection of fluorescent light (> 710 nm) was achieved using the probe of a Plant Productivity Fluorometer (Model SF-10; Richard Brancher Research Ltd., Ottawa, Canada). The prototype is described by Schreiber et al. [17]. The probe was supplied with a small cuvette containing saturated potassium dichromate solution as a filter for removal of blue excitation light, and the light-emitting diode was removed. The output signal from the SF-10 was stored in a Wavesaver Digital Waveform Recorder (Epic Instruments, Foster City, CA, U.S.A.; description by Wang and Murphy [18]). A change of time scale allowed recording of both the initial phase of the transient and the maximum value of fluorescence. Irradiation of the sample with blue light was kept to a minimum to preclude any risk of photoinactivation other than that intended. The relative number of photons reaching the outside of the cuvette was measured with an Optronics Model 742 Spectroradiometer calibrated against a standard lamp.

Experimental protocol

Thylakoid suspension, stored in liquid nitrogen, was thawed and kept in darkness 0°C for 1 h, and then diluted with the final resuspension medium. Forty μl of diluted suspension were transferred to the cuvette (1.92 μg Chl) and incubated in darkness for an initial 10 min before the fluorescence transient and the maximum

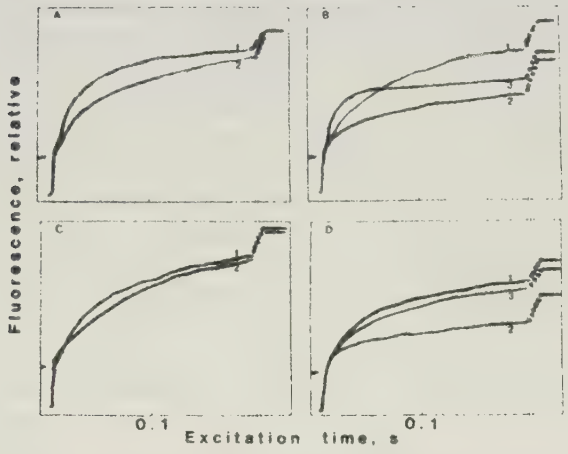

Fig. 2. Traces showing effects of UV-B radiation (6 min 280 nm, 12.6 μmol·m $^{-2}$·s $^{-1}$) on fluorescence induction in spinach thylakoids. A: Non-irradiated samples. Addition of 20 μM DCMU (1) shows that the excitation light was strong enough to cause virtually complete photoreduction of Q even in the absence of DCMU (2). B: Non-irradiated (1), 6 min 280 nm (2), 20 μM DCMU (in ethanol, final conc. 0.025%, v/v) added after irradiation (3). C: Non-irradiated (1), 20 mM hydroxylamine added (2). D: Non-irradiated (1), 6 min 280 nm (2), 20 mM hydroxylamine added after irradiation (3). F_0 is indicated on the y-axis.

fluorescence level (F_{max}) were recorded (controls). The sample was thereafter dark-incubated for 5 min before irradation with UV. The fluorescence transient and the F_{max} level after UV irradiation were recorded following a further 5 min dark period. The above procedure was followed for each wavelength in the UV range selected (248–340 nm, at 10 nm intervals). In order to check that the frozen and subsequently thawed thylakoids were still capable of O_2 evolution, the latter was measured using a Clark-type oxygen electrode (average of two experiments: 140 μmol $O_2 \cdot$ (mg Chl) $^{-1} \cdot h^{-1}$).

The following fluorescence parameters were determined from the recordings: initial fluorescence (F_0), F_{max}, F_v, and as a measure of the fluorescence rise time, the time required for the fluorescence to rise from F_0 to $F_0 + 0.75 \times F_v$. The various F values were determined on a relative scale. Results from two separate experiments were used for determination of an action spectrum showing the decline in the rate of fluorescence rise by UV radiation.

Hydroxylamine, an electron donor on the oxidizing side of PS II, was added to the thylakoid sample, and its effect on non-irradiated and irradiated samples compared (Fig. 2C, D). DCMU was used to ascertain whether the excitation light was of sufficiently high intensity to cause complete photoreduction of Q (Fig. 2A, B).

Results and Discussion

Fluorescence induction curves, reflecting photosynthetic electron transport, have characteristic patterns which change if the photosynthetic system becomes impaired, and can therefore be used as indicators of damage. With the method used the sample can be kept small and thin, enabling irradiation with intense UV of narrow band width and avoiding self screening.

In Fig. 2 original traces show the effect of UV irradiation on the fluorescence induction curve of isolated thylakoids. With the experimental arrangement used, the PS II excitation rate was very high compared with the rate of reoxidation of the PQ pool, and almost complete reduction of the PS II electron acceptor Q was achieved. This was shown by the fact that the F_{max} level was not increased by the addition of DMCU (20 μM; Fig. 2A). On the other hand, DCMU decreased the fluorescence rise time indicating that in non-poisoned thylakoids, the rate of electron transfer from Q to the PQ pool was comparable to the photochemical rate. In UV-treated thylakoids, the addition of DCMU increased the rate of fluorescence rise, but not F_{max}, which remained lower than the control level (Fig. 2B). This supports the conclusion by Iwanzik et al. [12] that the UV treatment increases radiationless de-excitation.

The balance between Q photoreduction and reoxidation provided a basis for a very sensitive indication of UV inhibition of electron transport through PS II. F_0 was hardly affected at all by the UV fluences employed, indicating that the chlorophyll was not photobleached. The amplitude of F_v was somewhat decreased by UV radiation. As an example, the amplitude of F_v was decreased by 8% by a photon

fluence of 12.6 μmol · m^{-2} · s^{-1} 280 nm radiation for 3 min, and by 19% when the irradiation time was 6 min. In contrast, the fluorescence time in non-poisoned thylakoids was increased by 101 ± 1% (average ± standard error, eight experiments) and 198 ± 1% respectively, by the two treatments. Representative curves for three wavelengths are given in Fig. 3. The large sensitivity of the rise time and the proportionality between increase in rise time and UV fluence applied make this an ideal indicator to use for the construction of an action spectrum for UV damage.

Since inhibitors on the water-splitting or oxidizing side of PS II reduce F_v and give a slower rise [19], the similar effect of UV radiation on PS II lends support to the idea of the lesion being on the oxidizing side of the reaction center, or possibly in the reaction center itself.

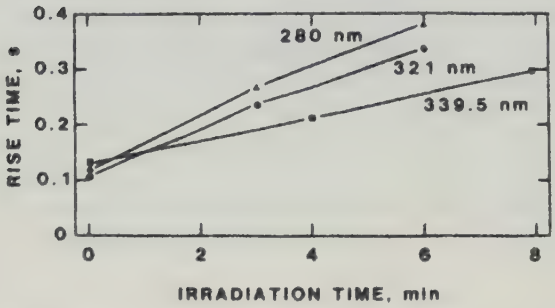

Fig. 3. Representative curves showing the increase in fluorescence rise time brought about by UV radiation (three different wavelengths). The rise time plotted is the time required for fluorescence to rise from F_0 to $F_0 + 0.75 \times F_v$.

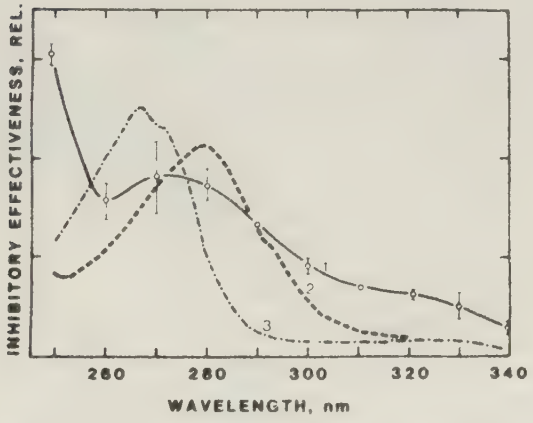

Fig. 4. Action spectrum for UV-induced increase in fluorescence rise time in spinach thylakoid membranes (curve 1); absorption spectrum of a 33 kDa protein isolated from spinach thylakoids [24] (curve 2); absorption spectrum of PQ (curve 3), redrawn from Crane [25]. The absorption spectrum for PQ is of the oxidized form in ethanol solution. Difference spectra for reduced-oxidized PQ in chloroplasts [26] show that the peak position is the same in chloroplasts as in solution. The reduced form has an absorption peak at 290 nm (not shown).

For determination of the action spectrum (Fig. 4, curve 1) the slopes of curves such as those in Fig. 3 were divided by the photon fluence rate. The action spectrum shows a small peak at 320 nm and a gradual increase to a peak around 275 nm. This was followed by a slight minimum at 260 nm and thereafter a sharp rise towards shorter wavelengths. The involvement of a protein as target molecule seems reasonable, although the identification of a specific protein is not possible at this stage. The 33 kDa protein isolated from spinach thylakoids was included for a representative protein absorption curve (Fig. 4).

The addition of hydroxylamine (20 mM) to thylakoid samples irradiated with 280 nm (12.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$) resulted in a partial restoration of F_v (Fig. 2D; cf. 2C). It is known that hydroxylamine is able to donate electrons on the oxidizing side of PS II. The fact that restoration of F_v is not complete may be due to the previously mentioned UV induced increase in radiationless de-excitation. It is therefore possible that the action spectrum shown in Fig. 4 is composed of more than one component. This possibility is under investigation.

Although the absorption spectrum for PQ (Fig. 4, curve 3; the spectra of PQ A, B and C are all reported to be similar [20]) overlaps to a certain degree the action spectrum in the UV-C region, the patterns of the curves are sufficiently dissimilar to exclude PQ as the single or main target of UV-C radiation. This dissimilarity is particularly pronounced in the 260–250 nm range, where the inhibitory effectiveness increases sharply towards shorter wavelengths as opposed to a decrease in absorp-

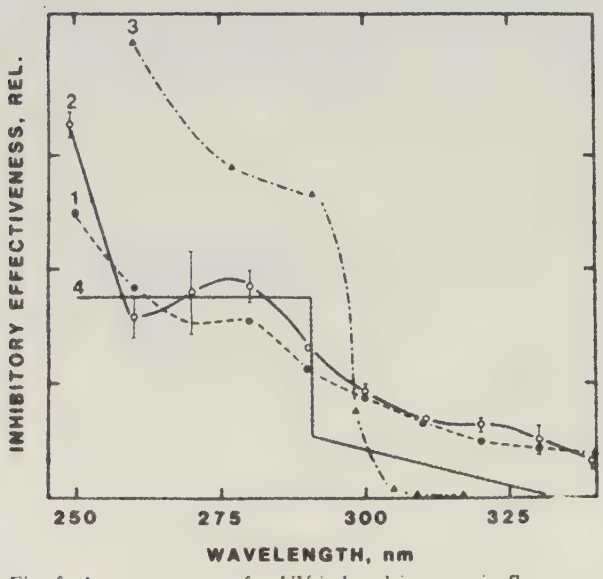

Fig. 5. Action spectrum for UV-induced increase in fluorescence rise time in spinach thylakoid membranes (curve 2) is compared with that of Jones and Kok [21] for the inhibition of photoreduction of dichlorophenolindophenol (PS II action) by isolated chloroplasts (curve 1), and with the action spectrum for inhibition of CO_2 uptake by *Rumex alpinus* (curve 3 [22]), as well as with the approximated action spectrum determined by Rundel [23] from values of M.M. Caldwell of the CO_2 uptake of *R. patientia* (curve 4).

tion of PQ. This agrees with earlier work by Shavit and Avron [5] and Mantai and Bishop [6], who found that UV-C does not destroy PQ in proportion to the inhibition of PS II function. The statement by Kulandaivelu and Noorudeen [10] that UV-C acts mainly at PQ is therefore not supported by our measurements.

Possibly the molecule(s) absorbing UV radiation is not identical to that which is damaged; there may be energy transfer from an 'antenna' to the sensitive site. In this case, the action spectrum for damage would reflect the absorption spectrum of the 'antenna'.

The action spectrum of Jones and Kok [21] of PS II inhibition in spinach chloroplasts is quite similar to that obtained for thylakoids using variable fluorescence in the present study (Fig. 5, curves 1 and 2 respectively). In contrast, Bogenrieder [22] obtained a somewhat different action spectrum for inhibition of complete photosynthesis using *Rumex alpinus* (Fig. 5, curve 3). Rundel [23], using unpublished results of M.M. Caldwell, determined an approximative action spectrum for inhibition of photosynthesis with polychromatic UV-B radiation, using the 'differential effectiveness method' (Fig. 5, curve 4).

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Stratospheric Ozone Reduction, Solar Ultraviolet Radiation and Plant Life
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Effects of Ultraviolet Radiation on Fluorescence Induction Kinetics in Isolated Thylakoids and Intact Leaves

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ABSTRACT

Action spectra were determined for the UV-induced delay of fluorescence induction (expressed as increase of half-rise time) in leaves of Elodea densa Casp. and Oxalis deppei Lodd. Elodea leaves showed increasing sensitivity with wavelength decreasing from 310 to 280 nm, while the Oxalis leaves, when irradiated from the abaxial side, gave a rather flat spectrum with lower sensitivity than Elodea throughout the range. When Oxalis leaves were irradiated from the adaxial side, UV sensitivity was even lower. The difference is partly explainable by a higher content of water-soluble, UV-absorbing substances in the adaxial epidermis. The action spectra are compared to that determined earlier for isolated spinach thylakoids. We also analyzed in more detail the effect of ultraviolet radiation on fluorescence kinetics at selected wavelengths. The fluorescence induction in isolated spinach thylakoids is the sum of three first order processes with different time constants. The main effect of ultraviolet radiation (280 nm) is to decrease the amplitude of the intermediate component. It also slightly retards the slow component. Results with 320 nm radiation were similar.

Fluorescence induction in Elodea leaves can be described as the sum of two first order components. The main effect of 320 nm radiation was to decrease the rate constant of the slow component.

The fluorescence rise in Oxalis leaves (and also in spinach leaves) has a biphasic pattern with a plateau in the middle. It is interpreted using a model with two kinds of photosystem II units: one set of unconnected units and one set with an energy transfer probability of about 0.65. The main effect of ultraviolet radiation is to diminish the fluorescence from the unconnected units, which have the largest rate constant.

INTRODUCTION

In another paper (Bornman et al. 1984) we described an action spectrum for the UV-induced decrease of the rate of fluorescence induction in isolated spinach thylakoids. In this chapter we analyze the kinetics of the induction more carefully. Spectral and kinetic analyses of the UV effects on the fluorescence induction in intact leaves of one aquatic (Elodea densa) and one terrestrial (Oxalis deppei) plant were carried out and internal screening effects were also evaluated.

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MATERIALS AND METHODS

Spinach (Spinacia olerace L.) was grown and thylakoids isolated as described elsewhere (Bornman et al. 1984). Elodea densa Casp. was purchased from a commercial supplier and kept in an aquarium at about 22.5°C. Illumination was about 40 μmol m^{-2} s^{-1} photosynthetically active radiation from a Philips TL F20W/33 fluorescent tube for 8 h per day, and the plants were kept under these conditions for at least a week before use. Oxalis deppei Lodd was grown in pots outside (unusually sunny and dry summer conditions in Lund, southern Sweden).

The apparatus and procedure for UV irradiation and fluorescence measurements are described in Bornman et al. (1984). Whole Elodea leaves were inserted into the water-filled quartz cuvette. From Oxalis leaves 3-mm wide strips were cut with razors and inserted into the air-filled cuvette. Before UV irradiation two control measurements were done on the unirradiated sample to ensure stable conditions.

The temperature of the cuvette holder was 10 $\pm$ 0.2°C for spinach thylakoids, 20.0 $\pm$ 0.2°C for Elodea and 18.0 $\pm$ 0.2°C for Oxalis leaves.

The influence of wavelength on the effect of UV irradiation was investigated for the leaves as previously for isolated thylakoids. Due to the amount of numerical work involved we have analyzed the spectral data in terms of amplitudes and rate constants of different kinetic components only at selected wavelengths, and for other wavelengths only recorded the half-rise time (the time taken for the fluorescence to rise from the initial (F_o) level to $F_o + (F_{max} - F_o)/2$ where F_{max} is the maximum fluorescence. It should be noted that the action spectrum for the effect of UV on spinach thylakoids described in Bornman et al. (1984) is based on the time to reach $F_o + (F_{max} - F) \times 0.75$, so allowance has to be made for this (by multiplication by the factor 0.659, computed from data in Table 1) when absolute sensitivities are compared.

In all cases the time was expressed as percentage increase over the control value for the same sample before irradiation, and this percentage increase was divided by the UV fluence to obtain ordinates for the action spectra. For Elodea leaves results for various irradiation times (i.e., for various fluences) were simply averaged. In the case of Oxalis the values tended to decrease with increasing fluence, and therefore a linear regression analysis (least squares fit) with extrapolation to zero fluence was carried out. In the case of Elodea we did not distinguish between irradiations from the adaxial and abaxial sides of the leaf, but in the case of Oxalis such a distinction was necessary as the leaves were much less sensitive to irradiation from the adaxial side. Water-soluble UV-absorbing substances were assayed in the following way: Epidermal strips were peeled from the abaxial and adaxial sides separately, air-dried to constant weight and extracted with quartz distilled water for about 1 h with occasional gentle agitation (no homogenization). The absorbance of the supernatant was recorded from 250 to 400 nm, after dilution when necessary and with quartz distilled water as reference.

RESULTS AND DISCUSSION

Fluorescence from the spinach thylakoids rose from the F_o level to the F_{max} level along a curve without any maximum or inflection point (Figs. 1A and 1B). The induction curve (F_{max} - F vs time) was the sum of three exponential (first order) components with time constants 840 ± 160 s^{-1}, 95 ± 13 s^{-1} and 23 ± 2 s^{-1}.

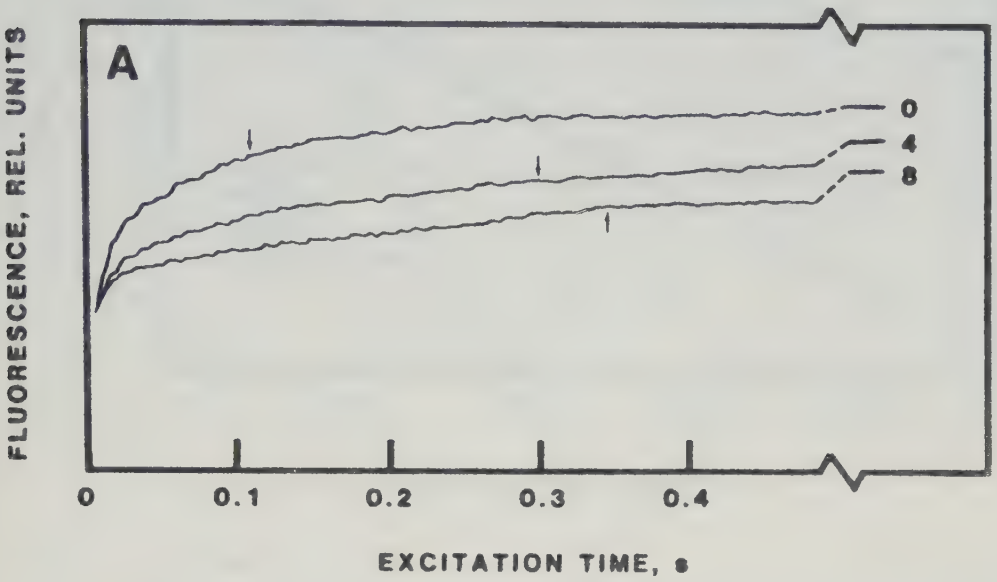

Fig. 1A. Typical recordings of fluorescence induction in spinach thylakoids. Numbers on the curves indicate minutes of exposure to 22.9 μmol m^{-2} s^{-1} of 320 nm radiation. The maximum fluorescence (F_{max}) is indicated to the right of the curves which start at the F_o level. The gap between the left frame and the start of the curves represents the opening time of the shutter. The arrows indicate the times required for 75% of the fluorescence rise.

The main effect of 280 nm radiation was a decrease of the amplitude of the intermediate component (Table 1). This occurred in such a way that the increase in time taken for the combined variable fluorescence components to rise to 75% of the maximum value was linear with UV fluence (linear with UV-irradiation time). The increase in time required to rise to 50% of the maximum value was, on the contrary not linear with UV fluence, but leveled off with increasing fluence. The effect of 320 nm radiation was qualitatively similar.

In the case of Elodea leaves the fluorescence induction (Figs. 2A and 2B) could adequately be described as the sum of two exponential functions. UV radiation (320 nm) decreased the rate constant, and to a lesser extent the amplitude of the slow component (Table 2). With 280 nm radiation the amplitude of the fast, but not that of the slow component decreased (results not shown).

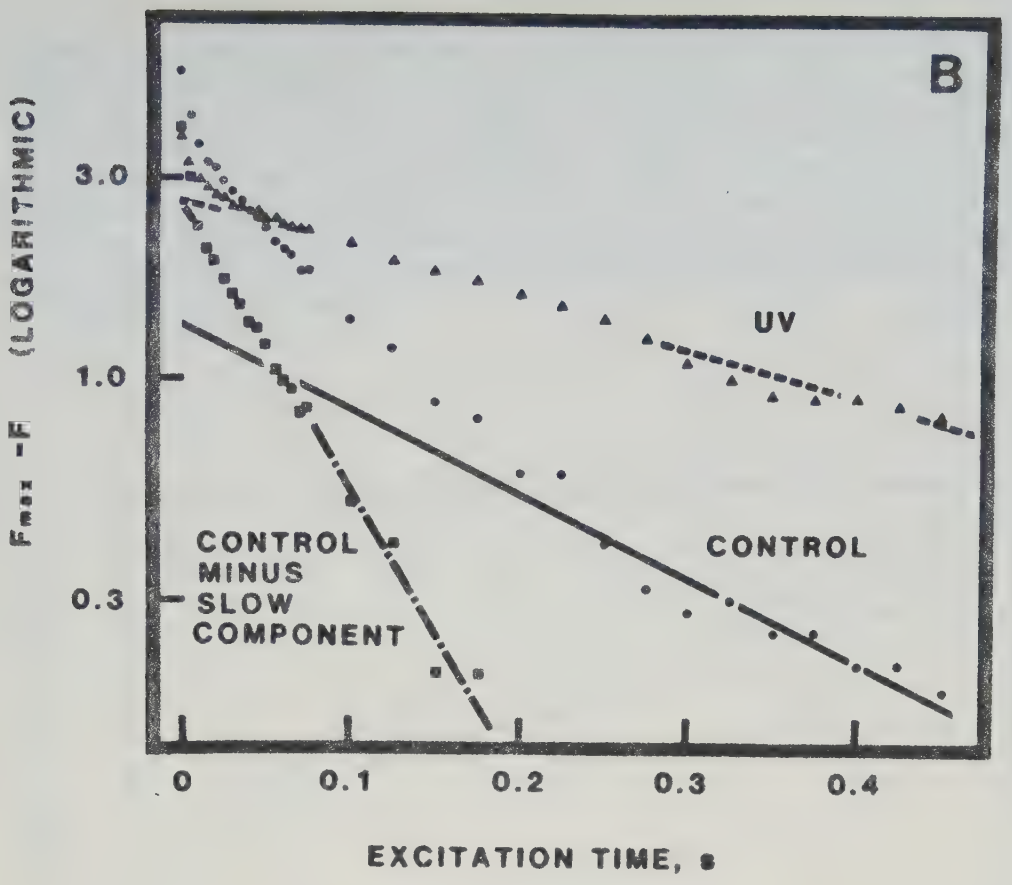

Fig. 1B. Semilogarithmic plot of data from Fig. 1A. Dots show data for non-irradiated (control) sample, and triangles data from sample treated for 8 min with 320 nm irradiation. The corresponding slow first order components are indicated by solid and dashed lines. Squares indicate the difference between measured control data and the solid line, and the dash-dot line is the intermediate first order component of the control (note that the first squares to the left lie above this line, indicating the presence of an even faster component). The intermediate component is practically absent from the UV-irradiated sample.

The fluorescence induction in Oxalis leaves exhibited more complicated kinetics. To show the changes over a wide time range we replotted the original recordings using a logarithmic time scale. In this way we could combine the results from 3 to 4 experiments in different time domains into a single diagram.

The fluorescence of Oxalis leaves (Fig. 3) rose rapidly from the initial (F_o) level to the intermediate level (I; cf. Govindjee and Papageorgiou 1971) where it leveled out. Somewhat later there was a second, slower rise to the F_{max} level, and then a biphasic decline. It was found that the blue light used for exciting fluorescence was strong enough to cause damage to the leaf when kept on for about the 100 s required for a curve like that in Fig. 3. Thus the final decline in fluorescence was partly an irreversible process. The "control" curve in Fig. 3 was recorded as second in a series of measurements; the variable fluorescence for the first control curve (not

shown) was about 40% larger. To avoid this damage during extended irradiation with blue light, it was decided to study more closely only the rising part of the curve and the effect of UV on this rise. This required only 2-s exposures to the blue light, which was not long enough to cause irreversible change. Nevertheless, Fig. 3 shows that the UV exposure employed (in this case 22.5 mmol m^{-2} of 340 nm radiation) did not eliminate the first phase of fluorescence decline, rather it only (together with the blue light exposure) decreased it in approximate proportion to the decrease in fluorescence rise.

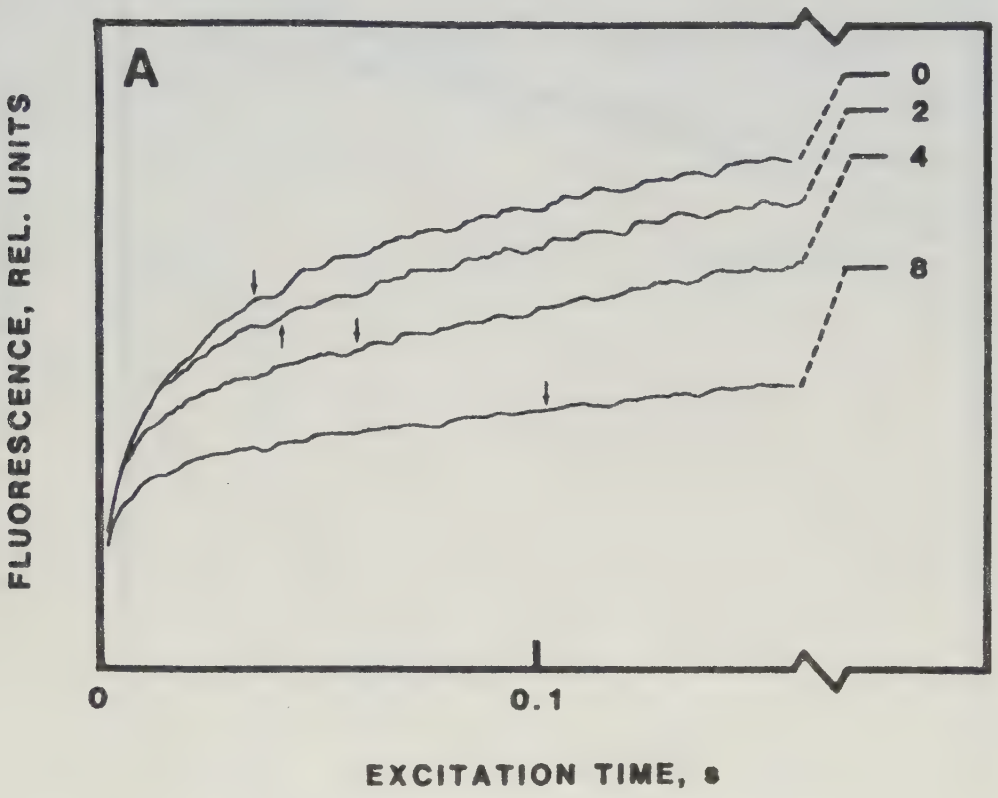

Fig. 2A. Typical recordings of fluorescence induction in an Elodea leaf. Numbers on the curves indicate minutes of exposure to 22.9 μmol m^{-2} s^{-1} of 320 nm, and arrows show half-rise times. Otherwise as for Fig. 2B.

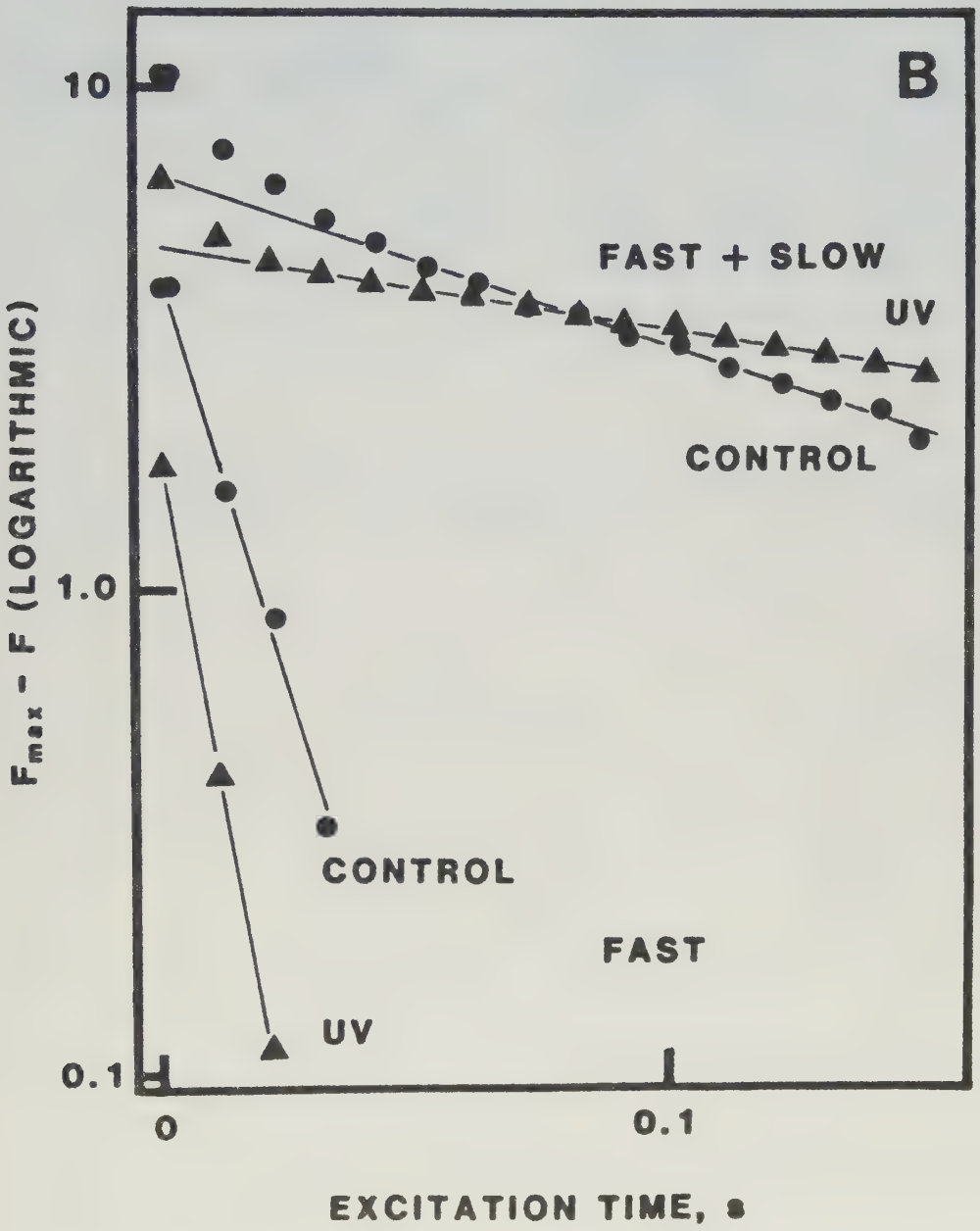

Fig. 2B. Semilogarithmic plot of data in Fig. 2A for 0 (control, dots) and 8 (triangles) minutes 320-nm irradiation (upper part of figure). The lines indicate slow first order component. Symbols in the lower part of the figure show difference between measured data and slow component.

Table 1. Effect of ultraviolet radiation (280 nm, 12.6 μmol m^{-2} s^{-1}) on amplitudes (A_i) and rate constants (R_i) of the three components of fluorescence induction in spinach thylakoids. The amplitudes are expressed as percentage of the average variable fluorescence of non-irradiated thylakoids, the rate constants in s^{-1}. Means and standard errors from seven experiments.

Irradiation time (min)	Fast component		Intermediate component		Slow component	
	A_1(%)	R_1(s^{-1})	A_2(%)	R_2(s^{-1})	A_3(%)	R_3(s^{-1})
0	27.2±3.3	840±160	45.6±5.3	95±13	27.2±2.9	22.9±1.5
3	23.1±0.7	793±55	16.2±2.7	75±4	38.1±1.7	17.4±0.7
6	21.4±1.4	853±50	5.0±2.4	84±14	34.9±4.3	13.2±1.1

Table 2. Effect of ultraviolet radiation (320 nm, 22.9 μmol m^{-2} s^{-1}) on amplitudes (A_i) and rate constants (R_i) of the two components of fluorescence induction in leaves of Elodea. Five experiments, otherwise as in Table 1.

Irradiation time (min)	Fast component		Slow component	
	A_1(%)	R_1(s^{-1})	A_2(%)	R_2(s^{-1})
0	33.5±5.7	129±21	59.8±6.8	13.2±2.9
2	36.0±3.9	97±21	46.0±7.3	6.8±1.3
4	33.0±4.8	108±28	42.8±6.0	5.2±0.8
6	35.5±9.4	93±21	40.0±6.2	3.9±0.7

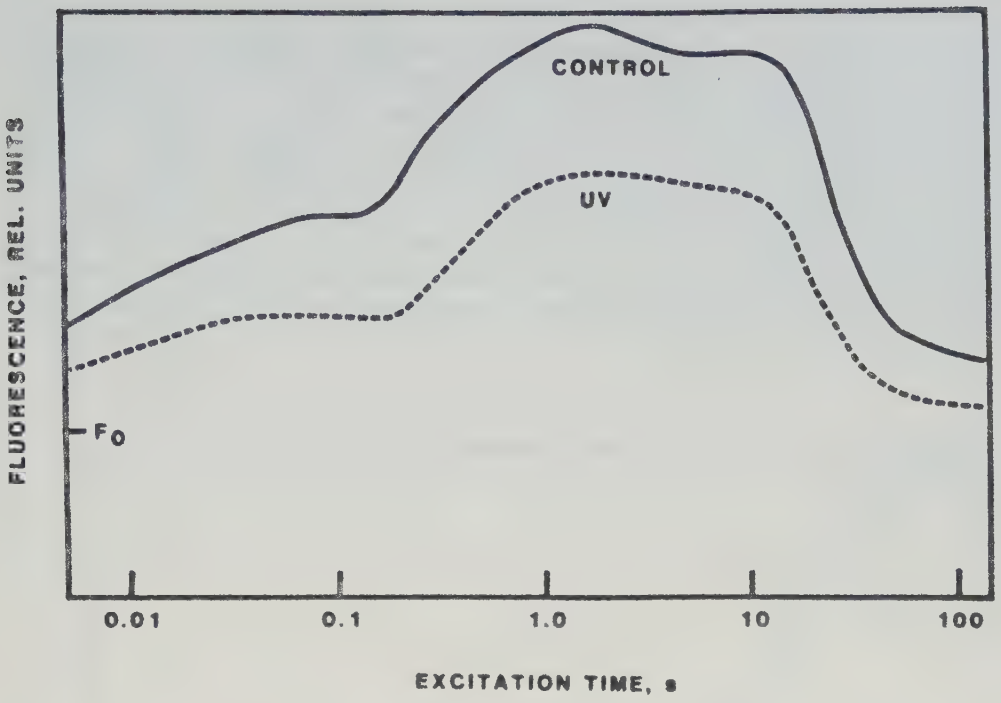

Fig. 3. Time course of fluorescence from an Oxalis leaf. The "control" was recorded as second in a series of measurements when the leaf had already been exposed to the strong fluorescence-exciting blue light (90 μmol m^{-2} s^{-1} centered at 440 nm) for 100 s. The first run with the dark-adapted leaf gave about 40% higher amplitude of variable fluorescence. The UV treatment consisted of 22.5 mmol m^{-2} of 340 nm radiation. Note the logarithmic time-scale. The magnitude of initial fluorescence (F_o) is indicated.

Figure 4 shows the fluorescence rise in an Oxalis leaf that had not been damaged by overexposure to blue light (dots). This rise can also be described as the sum of two components. In Oxalis however, contrary to spinach thylakoids and Elodea leaves, only the first component followed a simple exponential (first order) law. The slow component showed a lag. A similar pattern was found by Joliot and Joliot (1964) for the fluorescence induction in Chlorella. They explained this (also explained more fully by Lavorel and Joliot 1972) as being due to energy transfer between photosynthetic units. It seems that this explanation fits our experiments, and we therefore use their mathematical treatment, although other explanations have been advanced for such a lag. The model is strictly valid only for cells poisoned with DCMU to prevent reoxidation of reduced Q (electron acceptor of photosystem II). However, as Joliot and Joliot (1964) point out, if very strong exciting light is used (as is the case here), this makes very little difference in principle since reduction of Q is much faster than its reoxidation. Therefore we used the equation system of Lavorel and Joliot (1972) to simulate our experimental curves:

$$\phi_F = [(1 - p)\ (1 - Q)]/[1 - p(1 - Q)] \quad (1)$$

$$-p + (1 - p)\ \ln Q + pQ = R_2 t \quad (2)$$

In these equations ϕ_F is fluorescence yield, Q the proportion of photosystem II traps that are open, p the probability of interunit energy transfer, R_2 a rate constant and t time after switching on the exciting light. ϕ_F cannot be expressed as an explicit function of t, but since values of both ϕ_F and t can be computed for various values of Q, theoretical $\phi_F = f(t)$ relations could be plotted together with the experimental values in Fig. 4. For curve fitting the following five parameters could be varied: R_2, p and an amplitude factor A_2 for the slow component, and a rate constant R_1 and an amplitude factor A_1 for the fast component ($A_1 + A_2 = F_{max} - F_o$).

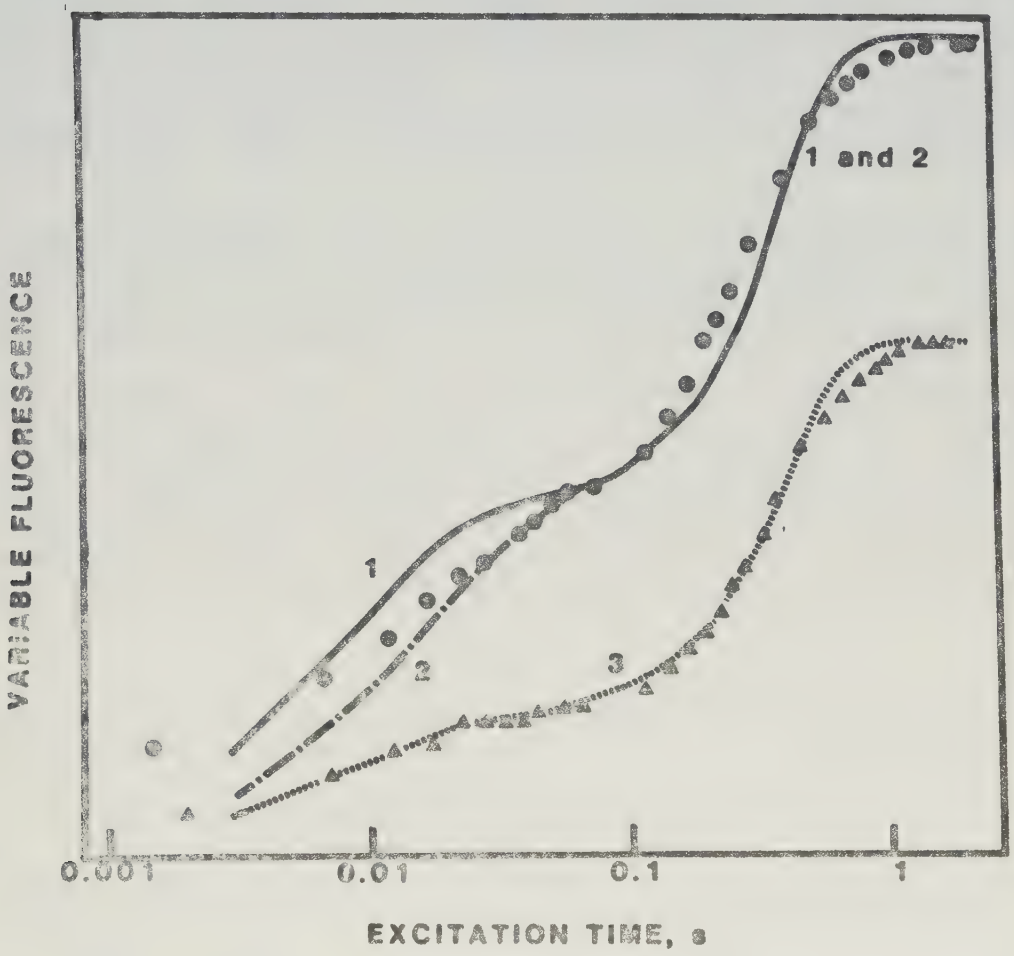

Fig. 4. Effect of UV radiation (11.7 mmol m^{-2} of 310 nm radiation) on the rising part of the fluorescence induction curve of an Oxalis leaf. In this case only brief exposures to blue light were used to avoid photoinhibition by blue light. Experimental values are denoted by dots (control) and triangles (UV irradiated); theoretical models 1, 2 and 3 (cf. Table 3) by solid, dash-dot and dotted lines, respectively. For times exceeding 0.1 s, models 1 and 2 coincide.

Our computing capacity did not permit, within reasonable time, an exact optimization of all parameters. We show two approximations for a non-irradiated leaf (Models 1 and 2 in Table 3) and one for the same leaf after exposure to a fluence of 11.7 mmol m^{-2} from 310 nm radiation (Model 3 in Table 1, cf. Fig. 4). Somewhat surprisingly, it seems that the apparent slowing down in this case is not due to a decrease of the rate constants of the slow component, but mostly to the fact that the amplitude of the fast component is decreased more than the amplitude of the slow one. The effect is distinctly different from the irreversible inhibition of the slow component brought about by extended irradiation with high-intensity visible light (Critchley and Smillie 1981). The latter authors have a different interpretation of the biphasic fluorescence rise than we do. According to them, the rapid component is due to electron transfer from photosystem II (and primary electron donors on its oxidizing side) to Q, while the slow component would represent electron transfer from the water splitting system, or intermediate electron carriers near to it. It is difficult to understand our results in the light of this model, since in our experiments the fast component is eliminated before the slow one. The fast reaction thus cannot be a prerequisite for the slow one as in the model of Critchley and Smillie. Besides, two consecutive reactions cannot result in a product curve with an inflection point, at least not if both reactions are first order.

As seen from Fig. 4, the final approach to the F_{max} level took place too rapidly with all models, as compared to the experimental data. This may be due to the fact that the samples were not DCMU poisoned and that Q reoxidation cannot be neglected when the reduction level is close to 100%.

We consider it likely that the two kinetic components found in Oxalis leaves are due to the existence of two kinds of photosynthetic units, or at least two kinds of photosystem II. The different rate constants could reflect a different antenna size. A possibility would be that one kind of photosynthetic unit is characteristic of grana thylakoids and one of stroma thylakoids. Another possibility is that the components correspond to the two kinds of photosystem II postulated by Arnon et al. (1981).

Table 3. Kinetic models for fluorescence induction in Oxalis leaves. All models follow the equation

$$F = F_o + A_1[1 - \exp(-R_1 t)] + A_2 \Phi_F$$

where t is time and Φ_F is defined by Eqs. (1) and (2), and with the following constants (A_1 and A_2 expressed as percentage of $A_1 + A_2$ for model 1).

Model	A_1(%)	R_1(s^{-1})	A_2(%)	R_2(s^{-1})	p
1	41.0	70	59.0	3.2	0.65
2	41.0	140	59.0	3.2	0.65
3	15.4	140	47.4	3.2	0.65

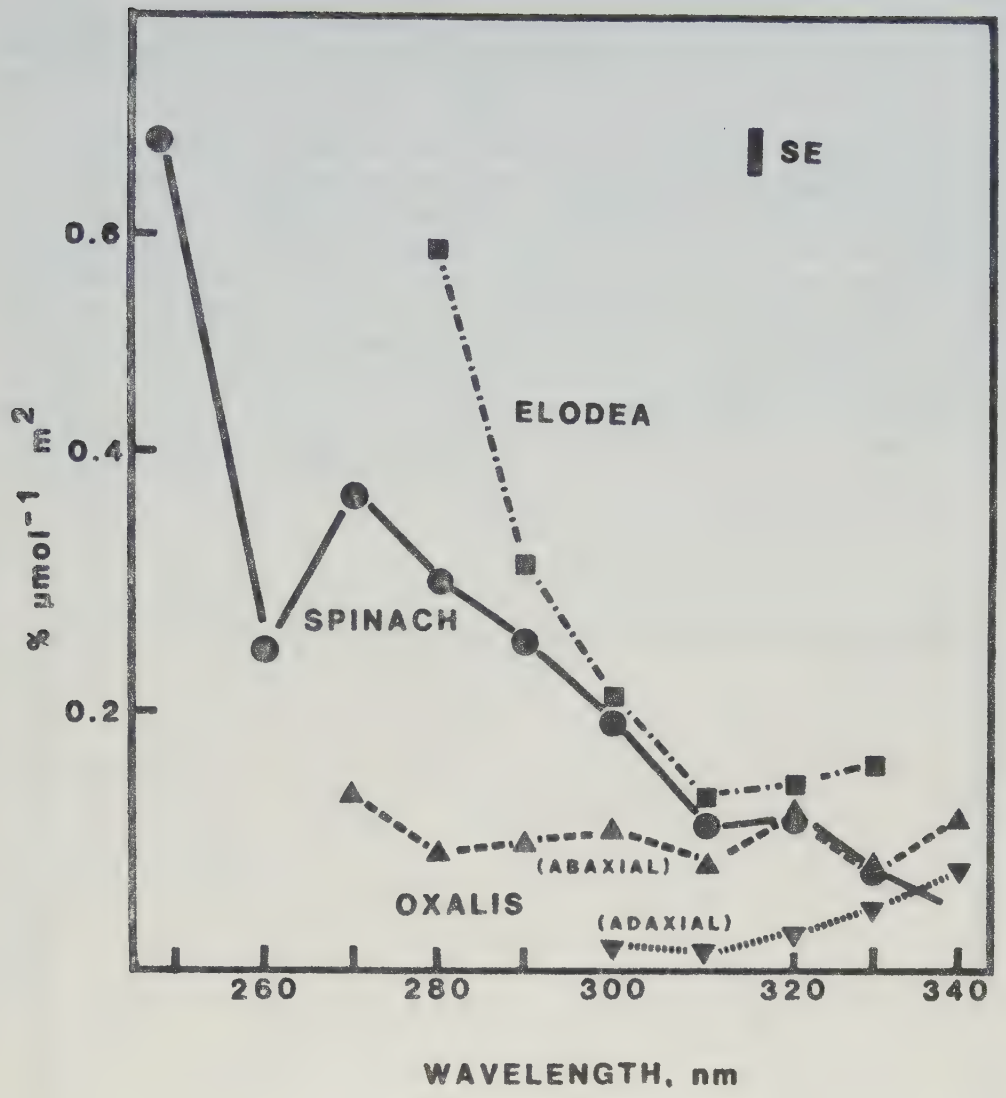

Fig. 5. Action spectrum for UV retardation of fluorescence induction (expressed as half-rise time) in leaves of Elodea and Oxalis. For comparison we also show a spectrum for UV retardation of fluorescence induction in isolated spinach thylakoids, recalculated from data in Bornman et al. (1984) and Table 1 to the same scale and averaged in the same way. The ordinate shows (percentage increase in half-rise time)/(μmol photons m^{-2}).

The half-rise time for irradiated leaves of Elodea and Oxalis was expressed as percentage increase over the control value for non-irradiated leaves. The results of the comparison of sensitivities to radiation of different wavelengths and radiation impinging on different sides of the leaves are summarized in Fig. 5. It is seen that the epidermis-free Elodea has the highest sensitivities throughout the spectral range investigated. Oxalis is more sensitive to radiation impinging on the abaxial side than to radiation impinging on the adaxial side. We suspected that larger amounts of water-soluble, UV-absorbing compounds were the cause of the difference. Extraction of excised epidermis and absorbance measurements on the extracts (Fig. 6) showed, on a weight basis, about 3 times as much absorbing

material in the adaxial epidermis. However, the absorbance maximum of the extracts is close to 330 nm, while the difference in UV sensitivity of the leaf is more pronounced at shorter wavelengths. Therefore, other substances than those easily extractable with water are likely to exhibit a more important protective role.

In conclusion it can be said that our spectral measurements have not yet led to an identification of the radiation sensitive target in photosystem II, but some compounds can now be excluded. The UV sensitivity of intact Elodea leaves is surprisingly high at 280 nm, and one can infer that even at the thylakoid level there are sensitivity differences between different plants. In the whole leaf the sensitivity is further modified by absorbing substances in the epidermis, and there may be large differences in sensitivity depending on the direction of the UV radiation in relation to a plant organ. The UV effects on fluorescence kinetics obtained with different plant materials cannot, at the present time, easily be interpreted under a unified model.

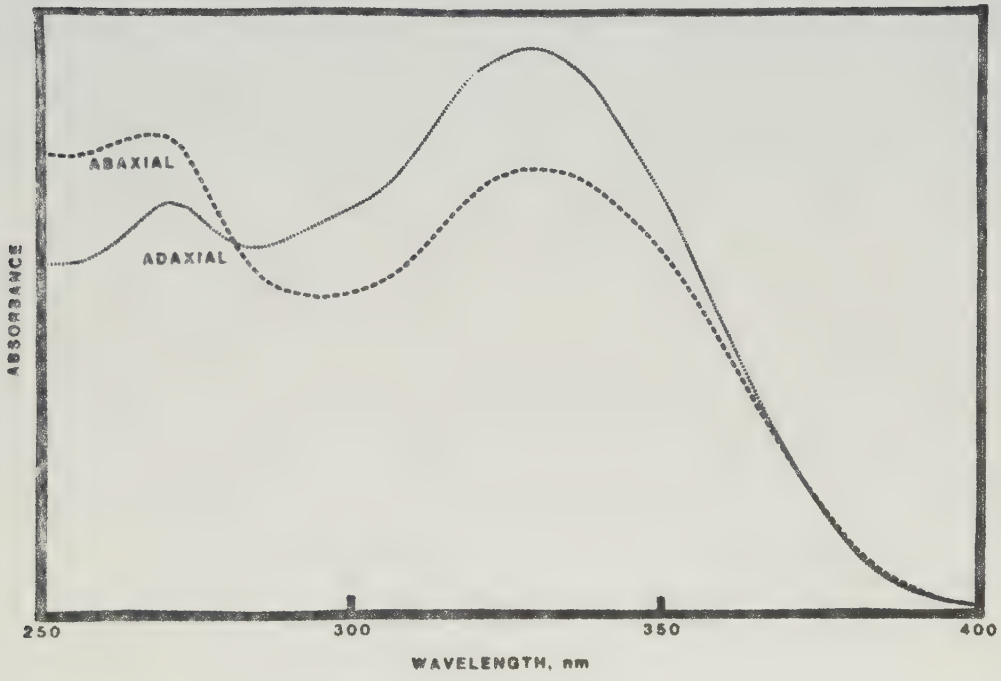

Fig. 6. Absorption spectra of aqueous extracts of the adaxial and the abaxial epidermis of Oxalis leaves. Different amounts of material and different instrument ranges were used; the maximum absorbance at 329 nm was recalculated to a concentration of 1 mg air-dried epidermis per ml extract, and corresponds to the following absorbances: adaxial epidermis 1.1 absorbance units, abaxial epidermis 0.39 absorbance units.

ACKNOWLEDGMENT

The authors gratefully acknowledge financial support from Nordiska Ministerrådet and Hilleshög AB.

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PRELIMINARY MANUSCRIPT

Inhibition of photosystem II by blue light and ultraviolet radiation: a comparison

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Summary

The effects of blue light (440 nm) and ultraviolet radiation (280 and 310 nm) on variable fluorescence in leaves of Oxalis deppei Lodd were compared. The effect of blue light, but not that of ultraviolet radiation (UV), was largely reversed during dark incubation. The amplitude of the slow component was markedly decreased by both irradiation with blue light and UV radiation. The rate constant of the fast component of the fluorescence induction curve was decreased by blue light. In contrast, the main effect of UV radiation was to increase the rate of the fast component.

Introduction

The recovery of the photosynthetic system from inhibitory high intensity visible radiation is well documented (see review by Powles 1984 and references therein). However, it depends on the intensity and duration of radiation as well as on whether the plants were grown under low or high light intensities. Recovery may be only partial with certain phases of the induction curve being more sensitive than the rest of the curve (Critchley and Smillie 1981).

It seems that neither UV nor high intensity visible radiation causes photobleaching of chlorophyll except at very high fluence rates. Photobleaching therefore is not the cause of photoinhibition of the electron transport chain, but may be a secondary process under extreme conditions (Powles 1984).

Certain similarities exist in the way in which high intensity visible and UV radiation affect plants. In both cases mostly PS II is affected and plants previously adapted to high intensity light are less sensitive than those grown under weak light (Critchley and Smillie 1981, Sisson and Caldwell 1976, Teramura et al. 1980).
In the present paper it is shown that despite these similarities, inhibition by visible (blue) and by UV radiation are two distinct phenomena.

Abbreviations: A_1, R_1, amplitude and rate constant of the exponential (fast) component of variable fluorescence; A_2, R_2, p, amplitude, rate constant and probability of energy transfer of sigmoid component according to Lavorel and Joliot (1972); DCMU, 3,-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{max}, maximum fluorescence; F_o, initial fluorescence, origin; F(t), fluorescence at time t after the onset of light; F_v, variable fluorescence; Q, first stable primary electron acceptor of PS II; Q_B, bound plastoquinone; PFD, photon flux density; PQ, plastoquinone; PS II, photosystem II; RC, reaction center.

Materials and Methods

Oxalis deppei Lodd plants, grown outside in pots under natural sunlight during summer, were dark-adapted for at least 1 h before use. For fluorescence measurements one leaflet was removed from the petiole and positioned against a quartz plate with either the abaxial or adaxial surface in the direction of irradiation. Measurements were carried out using a modified Aminco-Bowman spectrophotofluorometer as described previously (Bornman et al. 1984). Long wavelength fluorescence (> 710 nm) was measured. The temperature at the quartz plate was maintained at 20 ± 2 °C.

The fluorescence transient and F_{max} were recorded using excitation light at 440 nm (30 nm half band width, 90 μmol m^{-2} s^{-1}). For treatment with ultraviolet radiation,

280 and 310 nm (10 nm half band width, photon fluence of 17.7 and 27.6 mmol m^{-2}, respectively) were used, and for blue light treatment, 440 nm (30 nm half band width, 135 mmol m^{-2}). Irradiation time was 0 or 25 min for all treatments. In some of the experiments leaflets were also treated with DCMU (50 μM) by allowing a small cut surface, made at the base of the leaflet, to take up the DCMU solution (incubation time, 30 min). The effect of irradiation on the abaxial as compared to the adaxial side was also observed. Thin sections of leaves were made in order to view the anatomical structure. Preparation for light microscopy was carried out as described in the legend of Fig. 5.

For all treatments, values from the fluorescence induction curve ($F(t)-F_o$ vs time) were plotted on a logarithmic time scale. Values from at least two experiments were averaged and normalized to $F_v = 100$. This normalization was carried out in order to account for differences in light absorption and emission among leaves. Those values from treated material were then multiplied by the factor $[F_v \text{ (treatment)} / F_v \text{ (control)}]$ (except for Fig. 8).

The connected model of Lavorel and Joliot (1972) has its limitations. Nevertheless, experimental induction curves could be mimicked by those described by the model, and therefore it was used as a means for evaluating the changes caused by irradiation.

Results

Exposure of the abaxial side of Oxalis leaves to 25 min of blue light (440 nm) or to UV irradiation (310 nm) decreased the variable fluorescence (Figs 1,2). In the blue light-treated material the initial fluorescence rise appeared to be more inhibited than that of the UV-treated leaves over the same time range. There was a more pronounced plateau after UV irradiation (Fig. 2). After 30 min dark incubation the effect of blue light was nearly reversed. This marked restoration of the induction curve was not observed after UV irradiation.

Since it had been found previously that the adaxial side of Oxalis leaves was relatively insensitive to UV radiation (Björn et al. 1985), it was of interest to determine whether the insensitivity also held true for photoinhibition by blue light. After 25 min of UV irradiation (280 nm) to the adaxial leaf surface, no effect was observed (Fig. 3), whereas with blue light (440 nm) there was not only inhibition of the fluorescence rise, but dark incubation did not reverse the effect (Fig. 4) to the same extent as observed for the abaxial surface. Thin sections of the leaf (Fig. 5) showed unusually large epidermal cells, slightly closer packed on the adaxial surface. Below the upper epidermis are elongated palisade cells with deeper lying spongy mesophyll.

Lavorel and Joliot (1972) worked out their model for chloroplasts poisoned with DCMU, in which case no electrons flow from the primary quinone to the pool of free plastoquinone. We did a special series of experiments to study the effect of DCMU on fluorescence kinetics under our conditions of strong excitation light.

The seemingly small effect of addition of DCMU (Figs 6, 7) indicates that reoxidation of the primary quinone during the induction had only a minor influence on the fluorescence curves.

To better understand the effects of UV radiation on electron flow, the results of the experiments shown in Figs 6,7 with or without irradiation with 280 nm and with or without DCMU were further analyzed (Fig. 8).
For the DCMU-poisoned leaves, electron flow into Q (as well as the reduction level of Q, since no electrons proceed from Q to the free plastoquinone) can be computed according to the method of Murata et al. (1966) (cf. Malkin 1966, Melis and Homann 1976) from the integral $A(t) = \int_0^t (F_{max} - F(t))dt/F_v$ (not drawn in Fig. 8). The plot of this integral against $F(t) - F_o$ can then be used with the fluorescence values for the non-poisoned leaf to determine the Q-reduction level, (B(t)), corresponding to a certain fluorescence. On the other hand, the integral $C(t) = \int_0^t (F_{max}(DCMU) - F(t)(control))dt/F_v(DCMU)$ gives the electron flow through PS II in the case of non-poisoned leaves. The difference C(t) - B(t) then provides a

measure of the flow of electrons out of Q into the PQ pool. All values are related to the integral $A(\infty) = \int_0^\infty (F_{max} - F(t))dt/F_v$ for DCMU-treated samples (= 100). Since A(t) was directly determined only up to t = 475 ms, $A(\infty)$ was determined on the assumption that the final approach of A(t) to $A(\infty)$ was exponential. In Fig. 8 this is shown for non-irradiated leaves, as well as for leaves irradiated for 25 min with 280 nm radiation (11.8 μmol m^{-2} s^{-1}). Values are electrons per 100 active reaction centers (i.e. centers giving rise to variable fluorescence in DCMU-treated samples). To compare directly non-irradiated with radiated samples, one should remember that the variable fluorescence in the irradiated, DCMU-treated samples was only 71 % of that of the non-irradiated, DCMU-treated ones.

The standard error (SE) for F_v was <11 % of the mean value for all data points. As an example of the SE for $(F(t) - F_o)/F_v$ for the curves shown in Fig. 1, the following was obtained: control, SE was 2.1 % of the mean 0.03 s after the onset of excitation light and 1.5 % at 0.125 s; after irradiation with 25 min 440 nm, SE was 10.3 and 9.7 % at the respective times; and 3.2 and 0.1 % after 30 min dark incubation.

The fluorescence induction curve followed a similar trend as reported previously (Björn et al. 1985), in that it could be resolved into two components, one sigmoid and

the other first order. Therefore the same equations (8 and 9) of Lavorel and Joliot (1972) were used to add a sigmoid component to a first order component to obtain theoretical values which would best simulate those of the experimental results. Figure 9 A-C are examples of the curve fit obtained. The effect of the treatments were evaluated in terms of the amplitudes and rate constants of the fast and slow components of the fluorescence curve. This evaluation is presented in tabular form in Tab. 1. Results from a representative experiment where the abaxial surface was irradiated with 280 nm are included here for additional comparison.

With respect to the abaxial leaf surface of Oxalis, the rate constant of the fast component and the amplitude of the slow component were most affected by the UV photon fluences used (Tab. 1). The rate constant of the first component was decreased by blue light. It is of interest to note that Kyle et al. (1984) reported an increase (in the presence of DCMU) in the rate of fluorescence rise after photoinhibitory treatment with blue light.

As was also evident from the induction curves, there was little effect on amplitudes and rate constants by UV radiation on the adaxial surface of the leaf. Blue light, on the other hand, had a pronounced effect. After blue light inhibition the rate constants of both components did not show as much reversal after a 30 min dark period as those from the abaxial surface.

For the abaxial leaf surface, p (interpreted by Lavorel and Joliot, 1972, as the probability of exciton transfer between photosynthetic units) for UV-treated material increased towards unity. Blue light also resulted in an increase in p, which was reversible.

In non-irradiated material the effect of DCMU was to decrease p towards zero, which suggests that interunit exciton exchange was not occurring. In fact the units may always be separate on the pigment side, and apparent connectedness in non-poisoned material a result of backward electron flow from a common PQ pool. When DCMU was given after UV treatment the increase in p as a result of irradiation was largely reversed.

After UV irradiation of the adaxial leaf surface, exciton transfer between photosynthetic units did not appear to be affected, while after blue light treatment the same trend was followed as for the abaxial surface.

Discussion

It appears that blue light and UV radiation affect the fluorescence rise in different ways: the effect of blue light is largely reversed during a subsequent dark period, while that of UV radiation is not (Figs 1,2). Long exposure to very strong visible light may cause irreversible damage to the electron transport system of PS II, or allow only partial restoration (Critchley and

Smillie 1981). The irreversible inhibition by UV radiation does not seem to be of this kind, since the UV dose has been adjusted to give an inhibition level similar to (or less than) the reversible inhibition by blue light. Another difference between the effects of blue light and UV radiation is that the former decreases the rate constant (R_1) of the fast component, while UV radiation has an increasing effect. The increase could come about by the channeling of energy from antennae of blocked RCs (resulting in a decreased amplitude, A_1), to the remaining units.

The adaxial and abaxial leaf surfaces clearly exhibited different tolerance to UV radiation (Figs 2,3), which was probably due largely to more UV absorbing substances in the epidermis on the adaxial than on the abaxial side (Björn et al. 1985).

In contrast to the lack of effect of UV radiation on the adaxial leaf surface, blue light impinging on this surface markedly inhibited the fluorescence rise (Fig. 4). Reversal of this inhibition was not as complete as that for the abaxial side during the dark incubation used, but the reason for this difference is not known.

It should be pointed out again that the data in Fig. 8 are normalized to the integral $\int_0^\infty (F(t) - F_o)dt/F_v$ for DCMU-treated leaves, i.e. to photoreducible Q or active PS II RCs, which are different in non-irradiated and

irradiated leaves (in the same proportion as the variable fluorescence). One effect of UV radiation therefore, does not show up in this figure, viz. the complete switching off of a proportion of the reaction centers, which no longer give rise to variable fluorescence. This effect is what Renger et al. (1982) and Iwanzik et al. (1983) refer to as the creation of dissipative sinks. Figure 8 is designed to point out other effects.

The electron flow through PS II exhibits a smooth curve with gradually declining slope (Fig. 8, curves 1 and 2) and the rate is lower for the irradiated (curve 2) as compared to the non-irradiated leaves. This lower rate must be due to something else than to destruction of reaction centers ("creation of dissipative sinks"), and as a point of departure for further experimentation it may tentatively be assumed to be due to partial blocking of electron flow into the RCs. This latter effect could be identical to the hydroxylamine-reversible part of the UV-inhibited variable fluorescence found in (non-poisoned) spinach thylakoids.

Part of the electron flow through PS II proceeds beyond Q, presumably to PQ, but the rate of this flow shows variations (Fig. 8, curves 5,6). In the case of irradiated leaves it seems as if first one PQ pool is filled up, and then another one starts to fill up only after Q has reached a higher reduction level. For non-irradiated leaves the curve (5) looks more irregular, but shows a similar trend. At a Q reduction level of for

example, 50, fewer electrons have flowed over to PQ in the case of irradiated leaves, although a longer time has elapsed, and it also seems that the reducible PQ pools are smaller (Fig. 8, curve 6) in the case of irradiated leaves (despite that these pools are expressed in relation to photo-reducible Q). This points to some kind of ultraviolet effect also on the reducing side of PS II, possibly destruction of the Q_B-apoprotein (cf. Kyle et al. 1984), or of PQ. These guesses are very tentative, and further fluorescence induction experiments with UV of different wavelengths, with and without DCMU, as well as measurements with other techniques are required to separate these effects more clearly.

Although the general target area for both UV and high intensity visible radiation is known, there are differing opinions as to the exact target and mechanism of damage. Arguments in favour of either the oxidizing (see below) or the reducing side (Kyle et al. 1984, Renger et al. 1985) of PS II have been put forward. The oxidizing or water-splitting side has been advocated mainly on the basis that application of exogenous electron donors to P680 has not resulted in total recovery of electron transport rates, indicating that damage must have occurred on the oxidizing side or very close to the reaction center of P680 (visible light effect: Satoh 1971, Critchley and Smillie 1981; UV-B effect: Kulandaivelu and Noorudeen 1983, Smillie 1983, Bornman et al. 1984).

The results presented in this paper make it probable that UV affects the reaction center itself, as well as components on both the oxidizing and reducing sides of PS II.

Acknowledgements

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Figure legends

Fig. 1. Fluorescence induction curves from Oxalis leaves showing the influence of blue light (440 nm, photon fluence 135 mmol m^{-2}) and of 30 min dark incubation. The abaxial leaf surface was irradiated. ●, control; ○, blue light; ■, after 30 min darkness.

Fig. 2. The effect of UV irradiation (310 nm, photon fluence 27.6 mmol m^{-2}) on the fluorescence rise of the induction curve in Oxalis leaves. Otherwise for Fig. 1.

Fig. 3. Fluorescence transients from Oxalis leaves after exposure to 280 nm (photon fluence, 17.7 mmol m^{-2}) on the adaxial side. ●, control; △, UV radiation.

Fig. 4. The effect of blue light (440 nm, photon fluence 135 mmol m^{-2}) on the fluorescence rise in Oxalis leaves. The adaxial side was irradiated. ●, control; ○, blue light; ■, after 30 min darkness.

Fig. 5. Transverse section of an Oxalis leaf.
Leaf pieces were fixed in 6 % phosphate-buffered glutaraldehyde, dehydrated in methyl cellusolve and absolute ethanol, and embedded in glycol methacrylate. Sections were cut 3 um thick and stained with 0.05 % toluidine blue.
Bar = 16 µm.

Fig. 6. The effect of DCMU (50 µM) on the fluorescence induction in non-irradiated Oxalis leaves. ●, control; △, DCMU.

Fig. 7. Fluorescence induction curves from Oxalis leaves showing the effect of UV radiation (280 nm, photon fluence 17.7 mmol m^{-2}) and addition of DCMU (50 µM) after irradiation. The abaxial surface was irradiated. ●, control; ▲, UV radiation; △, after DCMU.

Fig. 8. Total electron flow through PS II (C(t) in text, curves 1 and 2), the amount of Q reduced (B(t) in text, curves 3 and 4) and the electrons flowing on from Q to PQ (or other secondary acceptors) (C(t)-B(t), curves 5

and 6), for non-irradiated *Oxalis* leaves and those irradiated with 17.7 mmol m^{-2} at 280 nm. Broken lines (odd numbers), non-irradiated; solid lines (even numbers), irradiated.
Curves drawn only for leaves not treated with DCMU.
Values computed as described in the text from the same experiments as shown in Figs 6 and 7. Vertical lines aid comparison of curves at a Q reduction level of 50.

Fig. 9 A-C. Fluorescence induction curves showing experimental data (o) and theoretical values (----) which were fitted using the equations of Lavorel and Joliot (1972). A, control; B, UV-treated (310 nm, photon fluence 27.6 mmol m^{-2}) and C, after 30 min darkness.

Table 1. The effect in Oxalis leaves of blue light (440 nm, 135 mmol m^{-2}) and ultraviolet radiation (280 and 310 nm, 17.7 and 27.6 mmol m^{-2}, respectively) on amplitudes (A) and rate constants (R) of the two components of the fluorescence induction curve. Amplitudes are expressed as percentage of the total variable fluorescence of the control. Rate constants are in s^{-1}. p, parameter, according to Lavorel and Joliot (1972), the probability of exciton transfer between photosynthetic units. Numbers in parentheses refer to the number of experiments. Irradiation time was 25 min in all cases and dark incubation after treatment, 30 min.
D, 30 min dark.

Treatment	Fast component A_1	Fast component R_1	Slow component A_2	Slow component R_2	p
ABAXIAL SURFACE					
Control[1)]	27	108	73	3.17	0.47
280 nm	21	140	41	2.50	0.80
D	24	140	46	2.26	0.82
Control[2)]	32	116	68	4.04	0.49
310 nm	29	216	52	3.13	0.73
D	30	160	55	3.28	0.74
Control[3)]	33	88	67	4.55	0.58
440 nm	22	68	40	3.36	0.70
D	30	80	64	4.00	0.60
Control[2)]	30	92	70	4.00	0.45
DCMU (50 uM)	36	140	74	3.88	0.09
Control[2)]	35	104	65	3.85	0.46
280 nm	22	120	44	2.50	0.60
DCMU (50 uM)	23	132	45	2.90	0.48
ADAXIAL SURFACE					
Control[2)]	21	160	79	3.77	0.60
440 nm	10	92	59	2.40	0.80
D	17	136	77	2.63	0.60
Control[1)]	22	160	78	2.63	0.62
280 nm	22	160	77	2.61	0.60

FIG 1

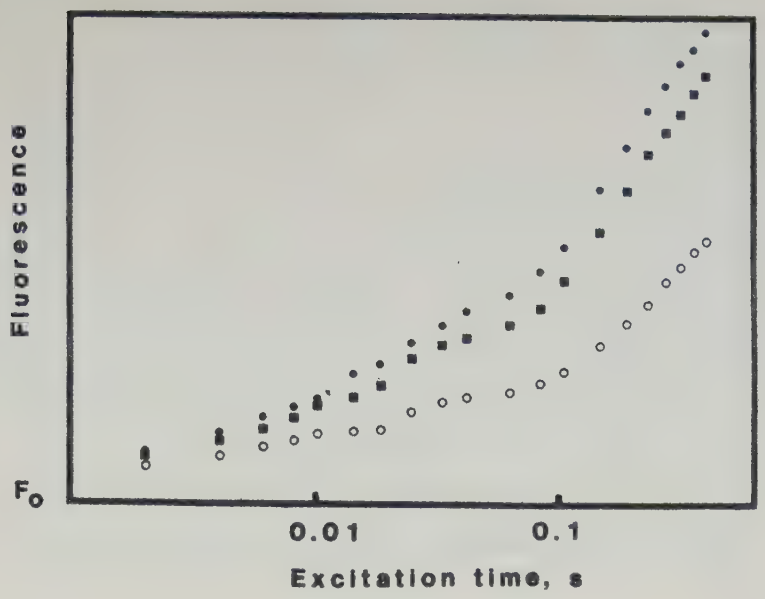


FIG 2

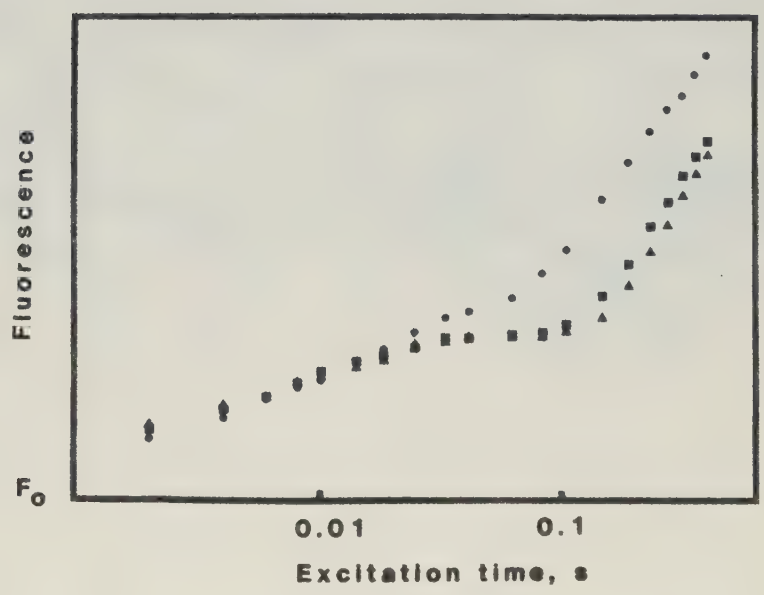


FIG 3

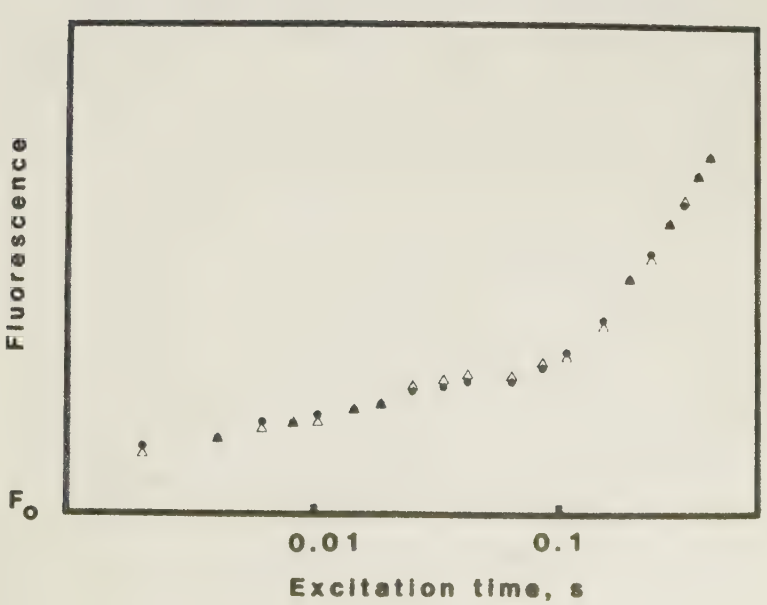


FIG 4

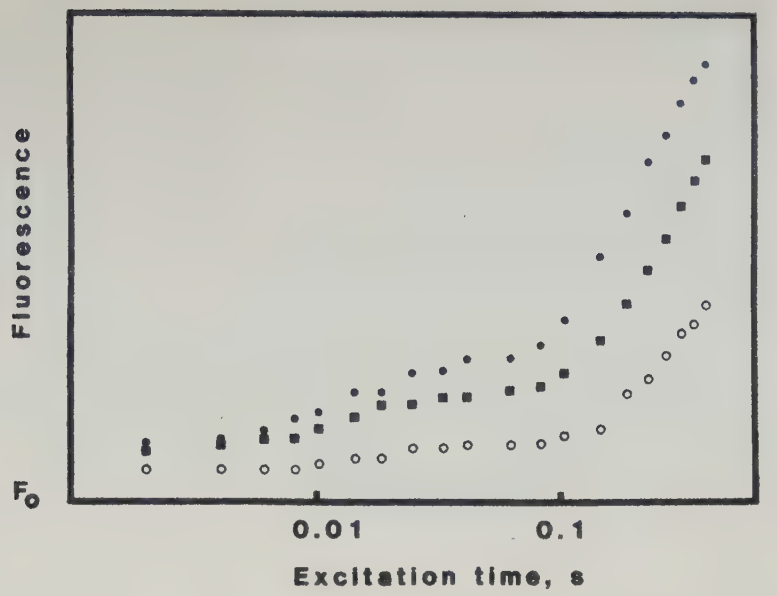


FIG 5

FIG 6

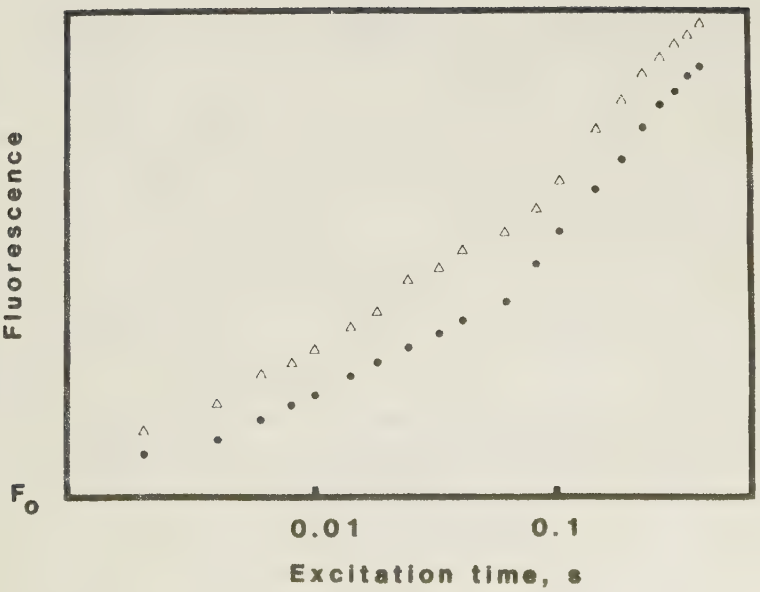


FIG 7

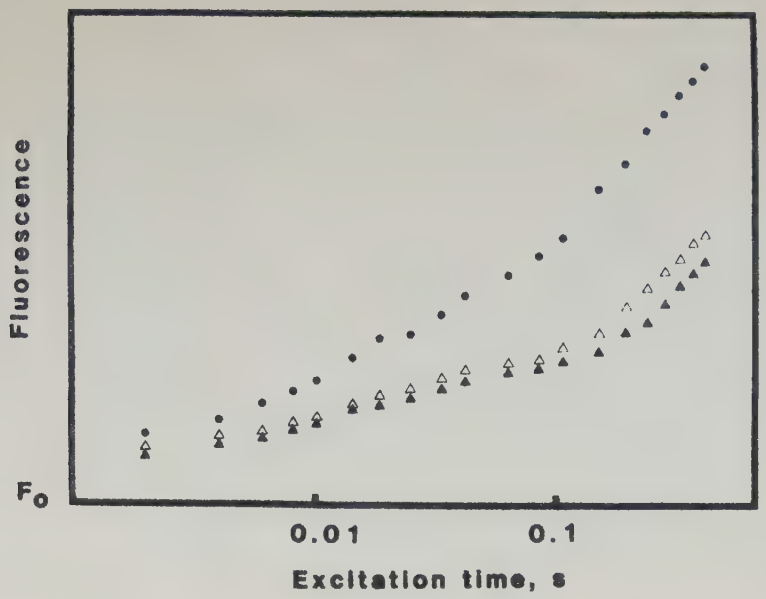

Fluorescence
F_o
0.01
0.1
Excitation time, s

FIG 8

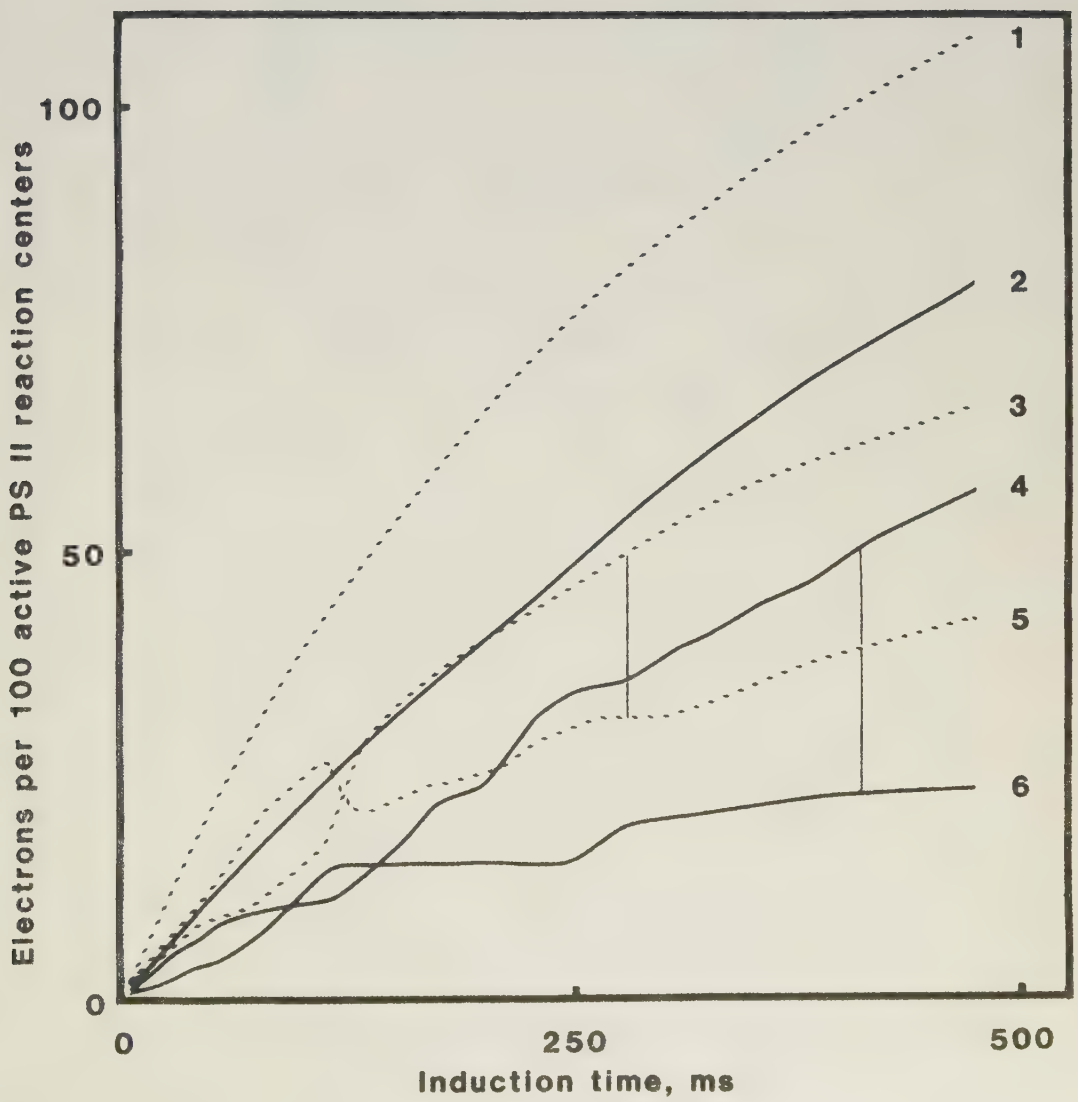
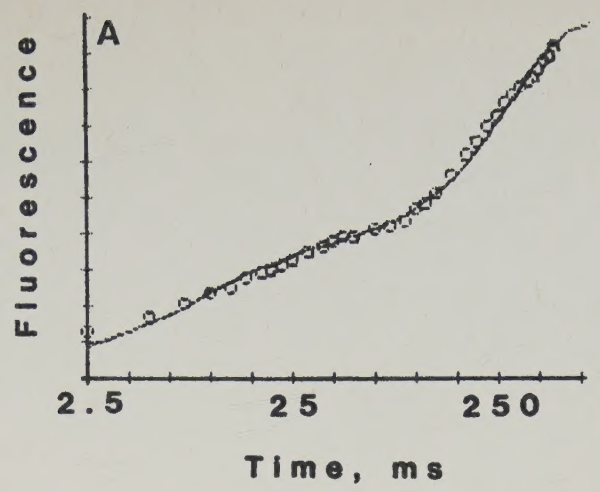
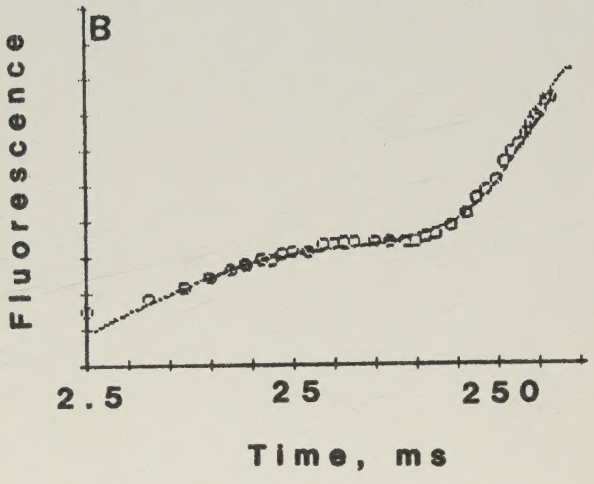
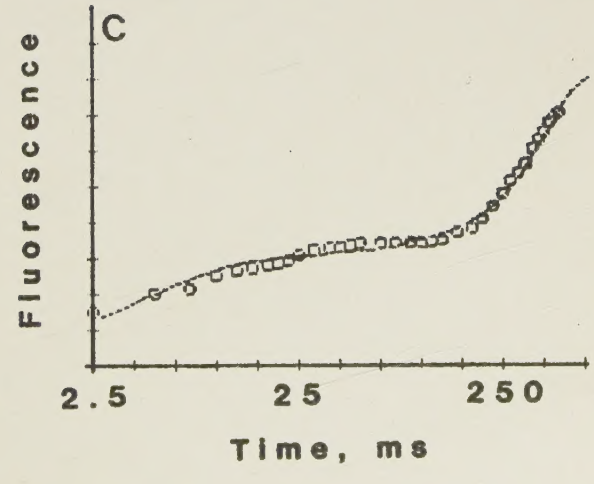

100
50
0
Electrons per 100 active PS II reaction centers
1
2
3
4
5
6
0
250
500
Induction time, ms

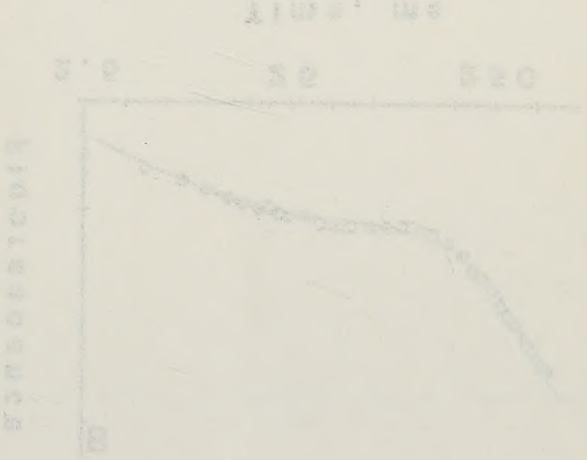
FIG 9A-C

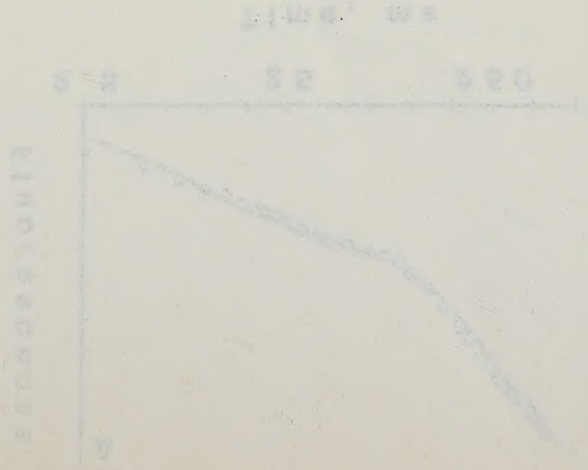

A
Fluorescence
2.5
25
250
Time, ms


B
Fluorescence
2.5
25
250
Time, ms


C
Fluorescence
2.5
25
250
Time, ms

Fluorescence
C
Time, ms


Fluorescence
B
Time, ms


Fluorescence
A
Time, ms